

**Osmotic balance and establishment of polarity in the  
*C. elegans* embryo require cytochrome P450 CYP31A**

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## 1- SUMMARY

Lipids carry out important structural as well as signaling functions in the cell. In recent years, enzymes that metabolize lipids have been emerging as key regulators of basic cellular functions and developmental processes. In order to study metabolism of lipids, we have focused our research on a class of proteins: the cytochrome P450s (CYPs), which are involved in lipid production in many organisms.

We have used *C. elegans*, a classical genetic model system, to investigate lipid metabolism because this nematode offers several technical advantages that render it suitable for our investigations. The aim of our project was to identify and characterize essential lipids for the development of worms. We have performed RNAi (RNA interference) against *C. elegans* CYP31A, and found that silencing of this enzyme leads to the arrest of embryonic development. Further characterization of this embryonic lethal phenotype revealed that it is caused by problems in establishment of polarity and failure in the extrusion of a polar body. Moreover, we found that embryos depleted of CYP31A are osmotic sensitive and their eggs are permeable to dyes (hoechst, FM 4-64 *etc.*). The defects described above are common to a class of mutants that received the denomination of POD (for Polarity and Osmotic Defects). Analysis by electron microscopy demonstrated that *cyp31A(RNAi)* embryos exhibit an improperly constructed eggshell. Further functional studies have demonstrated that the defects observed in *cyp31A(RNAi)* embryos can be ascribed to the malfunctioning of one of the three layers of the eggshell: the lipid-rich layer, but additional problems in the assembling of the other two layers are also present.

In order to identify the product of CYP31A, we set up a bioassay in which we tested the capability of lipidic extract from wild type embryos to rescue the embryonic lethality. The bioassay provided a method to track the activity and allowed us to enrich the metabolic product of CYP31A by the fractionation of the total lipid extract.

Another POD gene, *emb-8*, codes for an NADPH CYP reductase. This



protein supplies electrons to the CYPs for their metabolic reactions. A mutant of *emb-8* (*emb-8(hc69)*), gives a similar phenotype as the knock-down CYP31A.

With the aim to test if EMB-8 and CYP31A act in the same pathway we extracted lipids from *emb-8<sup>TS</sup>* mutants. We tested in the bioassay if extracts from *emb-8(hc69)* mutants, containing the metabolic product of CYP31A, can rescue *cyp31A(RNAi)* phenotype. The results obtained suggest that EMB-8 and CYP31A work in the same metabolic pathway.

Conclusively, CYP31A and EMB-8 cooperate to produce a class of lipids that are required for the construction of a functional eggshell. A defective eggshell causes failure in polarity establishment, extrusion of the polar bodies, osmotic sensitivity and permeability and eventually it leads to the arrest of the development of *C. elegans* embryos.

*“It is better to add life to the days than days to life”*

Rita Levi Montalcini (Italian Nobel prize laureate)

## 2- INTRODUCTION

### 2.1 Lipids

Lipids comprise diverse classes of compounds that fulfill crucial roles in the cell. Their functions can be divided into “structural roles” (in which they are used to assemble cellular structures such as membranes); and “signaling roles” in which lipids directly or indirectly affect the communication within or among cells. In order to accomplish this ample range of functions, the lipid structures have been widely modified during evolution, so that nowadays more than 1000 different forms of lipids exist (van Meer G., 2005). Lipid modifications affect their molecular structure in numerous ways. In the class of phospholipids, for instance, the molecules that constitute the head groups can greatly vary. Furthermore, each hydrophobic tail can have a varying length and degree of unsaturation. All these modifications can differentially affect the physiological behavior of the phospholipids in the cell (Guo Z. *et al.*, 1989).

*-Lipids form dynamic structure such as membranes or resistant construction such as cutin*

Lipids constitute about 50% of the mass of most animal cell membranes. There are approximately  $5 \times 10^6$  lipid molecules in a  $1 \mu\text{m} \times 1 \mu\text{m}$  area of a lipid bilayer (Alberts B. *et al.*, 2002). All the lipids in cell membranes are amphipathic. Therefore, based on their chemical-physical property, lipids in membranes spontaneously self-assemble ordered layers juxtaposing the hydrophobic poles of their molecules to exclude water from the internal part. The double lipid layer of membranes represents the energetically most convenient configuration. The spontaneous creation of this double-layered structure and the compartmentalization turn out to be decisive for the existence of the cell. Membranes not only isolate the cell from the outer environment and define its borders, but they also confine the different organelles and compartments that permit the cell to accomplish the functions required for its existence. When components of a cell are enclosed into

membranes, the products of enzymatic reactions cannot diffuse away but are contained within the determined space where they could be used (Berg J.M. *et al.*, 2002). Furthermore, lipids not only constitute the mere structure of the membranes in a static manner, but by their dynamic actions and different composition, lipids influence membrane properties such as fluidity and ion permeability (Haines T.H., 2001).

Different classes of lipids, together with specific proteins (RABs and SNAREs), confer specific identity features to the membranes of different sub-compartments of the cell. The fact that membranes differ in respect to their thickness and composition has been observed a long time ago; however, the way these differences are kept through biosynthesis, remodeling, hydrolysis and transport has not yet been completely elucidated.

The dynamic behavior of lipids in membranes resides in the weak interactions among them (hydrogen bonding at the lipid/water interfacial region, hydrophobic interactions, and van der Waals interactions) (Mukherjee S., Maxfield F.R. 2000). However, when lipids are covalently polymerized, they can also construct very resistant and rigid structures such as cutin or suberin in plants. This class of lipids has an essential function. Cutin, for instance, is a biopolyester of esterified hydroxylated fatty acids involved in waterproofing the leaves and fruits of higher plants and regulating the flow of nutrients and minimizing the impact of pathogens. (A. Heredia 2003; P.E. Kolattukudy *et al.*, 1995; 2001).

#### *-Lipid rafts: structural role influences signaling functions*

Specific lipids can affect the distribution of the proteins in the membrane by the constitution of “lipid rafts”: specialized microdomains in the membrane lateral plane (Simons K. and Ikonen E., 1997; Rajendran L. and Simons K. 2005). In these areas, specific lipids (mainly sphingolipids and cholesterol) accumulate based on their chemical-physical properties and because of this, certain membrane proteins can be specifically sorted in these structures. Lipids in these areas (ordered phase) confer different biochemical characteristics from the ones of the surrounding (disordered phase) such as, for instance, being resistant to cold detergent extraction. (Rietveld A. and Simons K., 1998) Lipid rafts have received considerable attention because

they are thought to be involved in signal transduction and cellular polarization (Harder T., 2004).

### *Signaling role*

Besides their structural role in membranes, a single lipid can activate transcription factors when it acts as a hormone or a secondary messenger (Alberts B. *et al.*, 2002). In this way lipids can regulate and modulate the metabolic and developmental programs of cells. For instance, steroids can diffuse through the membrane into the cytoplasm of target cells where they can bind to their specific receptor. The receptor is inactive, being associated with a Heat Shock Protein (HSP). The HSP is released after the binding of the hormones (Lange C.A. 2004). Now, two receptors can dimerize, move into the nucleus, bind specific DNA sequences and induce the transcription of target genes (Pratt W.B. and Toft D.O., 2003).

In addition to this “direct” function, lipids may influence intercellular signaling through a different mechanism: specific enzymes can covalently attach lipids to proteins, thus altering their cellular localizations. In many cases, the lipid-anchoring modification is essential for the function of a protein: many membrane proteins are anchored to the membrane by a lipid moiety (e.g., the glycosylphosphatidylinositol or GPI-anchored proteins) (Hoessli DC & Robinson P.J., 1998), cholesterol (e.g., hedgehog) (Mann R.K. and Beachy P.A., 2000; Ingham P.W. and McMahon, A.P., 2001; Beachy P.A. *et al.*, 1997), a fatty acid such as myristoyl or palmitoyl (e.g., Src family kinases) (Resh M.D. 1994), or an isoprene unit (e.g., small GTP-binding proteins) (Zhang F. L. and Casey P.J. 1996).

## **2.2 The model system *Caenorhabditis elegans***

In spite of the importance of lipids in terms of signaling and structure, only a limited number of lipids and pathways have been characterized at the molecular level. The problems usually encountered are associated with the scarcity of technical tools for unraveling and identifying the molecular structure of these small molecules. Moreover, interpretation of protein knock-down experiments is not always easy.

One way to simplify the dilemma of various functions of lipids could be by using the nematode *C. elegans* as an investigation tool. This worm is a classic genetic model system (Brenner S., 1974), which has, however, recently received novel attention as a valuable organism for studying the orchestration of lipid metabolism and function. *C. elegans* undoubtedly presents numerous technical advantages as a model organism. I) It has a short life cycle: it needs 3 days at 25° C degrees to become a fertile adult; II) it produces a large brood size (300-350 self progeny) (Wood W.B., 1988); III) the embryos are transparent and rather big (about 50 micrometers) allowing the observation of cellular development and fate by DIC (Differential Interference Contrast) microscopy.

*C. elegans* has been considered as a valuable model organism also for clinical studies because the major cellular metabolic pathways are conserved across the phyla. It has been predicted that about 75% of human disease genes have a *C. elegans* homologue (Kuwabara P.E. and O'Neil N., 2001; Kaletta T. and Hengartner M. O., 2006).

### **2.3 Genetic features and techniques**

*C. elegans* was the first multicellular organism for which the entire genome was sequenced (1998, later reviewed in 2002). This nematode genome is composed by  $100 \times 10^6$  base pairs and has been predicted to contain 19.000 genes (<http://wormbase.org/>) distributed along five pairs of autosomic and one sexual chromosome. In 2003 also the sequences from the genome-program of related nematode *C. briggsae* were published, allowing researchers to study and compare these two evolutionarily related nematodes.

A long list of *C. elegans* mutants (deletion or point mutations) has been generated by chemical mutagenesis, irradiation or by a combination of both (Anderson P., 1995; Moerman D.G. and Baillie D.L., 1981; Johnsen and Baillie 1988; Stewart H.I. *et al.*, 1991; Yandell M.D. *et al.*, 1994). In addition, *C. elegans* offers the possibility to create transgenic lines that expresses fluorescent proteins. This is an useful tool to investigate subcellular localizations of proteins (Miller D.M. 3<sup>rd</sup> *et al.*, 1999).

*C. elegans* exhibits sexual dimorphism: male (sexual chromosomes X/0) and hermaphrodite (X/X) that have 1031 and 959 somatic cells respectively (Riddle D.L. *et al.*, 1997). The males occur in nature by meiotic nondisjunction of the sex chromosomes. This phenomenon increases in case of stress (changed environmental conditions) and helps to introduce a higher genetic diversity. The variability introduced by the males could potentially generate forms more adapted to the new environmental situation. In standard conditions, only one male per 500 laid eggs is produced (Wood W.B., 1988). The sexual dimorphism allows the researcher to perform classic crossing experiments in order to create new lines of *C. elegans* that combine parental mutations and/or transgenic lines that express fluorescent proteins.

In addition to the mutants collected throughout the years, the establishment of the RNAi (RNA interference) technique that allows the knock down of a gene, has opened a new age of functional genomics. RNAi was first discovered in *C. elegans* (Fire A. *et al.*, 1998). So far, four methods are reported for the delivery of double-stranded RNA (dsRNA) in *C. elegans*: I) injection of dsRNA into the animal (Montgomery M.K. and Fire A., 1998), II) feeding with bacteria that produce dsRNA (Timmons L. and Fire A., 1998), III) soaking in liquid media with dsRNA (Tabara H. *et al.*, 1998), IV) *in vivo* production of dsRNA from transgenic promoters (Tavernarakis N. *et al.*, 2000). Using these powerful genetic tools, several genome-wide screens with different methods and in different conditions have been already carried out. (Gönczy P. *et al.* 2000; Fraser A.G. *et al.*, 2000; Maeda I. *et al.* 2001; Piano F. *et al.*, 2002; Ashrafi K. *et al.*, 2003; Simmer F. *et al.* 2003; Kamath R.S. *et al.* 2003; Sönnichsen B. *et al.*, 2005). In contrast to mammalian system where siRNA should be short and specific to avoid the activation of the interferon response, in *C. elegans* immune response triggered by long dsRNA has never been detected rendering the system more suitable for genome-wide screening approaches.

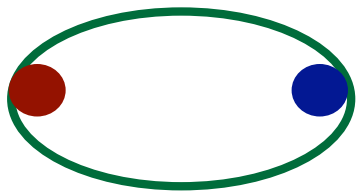
## **2.4 Development of *C. elegans***

Since we aim to characterize lipids that are essential for the development of *C. elegans*, it is important to give a brief description of the

strict developmental program carried out in this nematode. *C. elegans* has an invariant plan of development: a regulated pattern of cell divisions produces diversity of fates among the blastomeres. The different cells created in this way will be the precursors of the distinct tissues that will compose the organism. Development starts when the oocyte is fertilized passing through the spermatheca (the organ that stores spermatozoa). After the penetration of the sperm into the oocyte, a cue is sent to the female pronucleus, that before fertilization was arrested in meiotic prophase I. This stimulus causes resumption of meiosis (fig. 1A) (Miller M.A. *et al.*, 2001). The pronucleus of the oocyte goes through two anastral meiotic segregations that eventually lead to the extrusion of the polar bodies from the anterior side of the embryo-cortex. Thereafter, the two haploid pronuclei are ready to begin the process that will lead to the creation of a multicellular organism (fig. 1 B, C). The female pronucleus moves toward and reaches the sperm pronucleus. Then, it starts the fusion of the two (haploid) chromosomal sets to create the first zygotic nucleus. Eventually, the embryo (fig. 1D-F), accomplishes the first cytodieresis (cellular division) generating two asymmetric embryonic daughter cells: a larger anterior called (AB) blastomere and a smaller posterior (P1) cell (fig. 1 G, H).

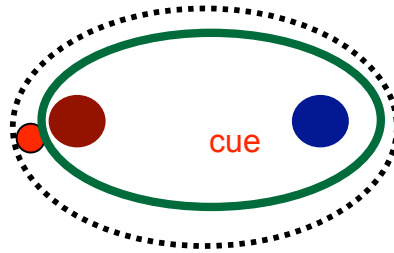


Fig.1



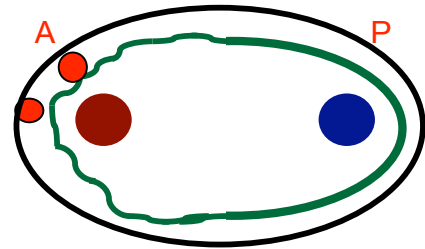
**A:~0'**

Fertilization: the sperm (blue) enters at the posterior pole. Anterior-posterior polarity process is initiated.



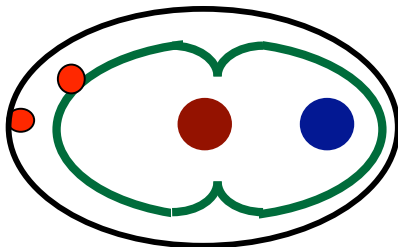
**B:~10'**

Rapidly, an eggshell (black line) is synthesized. The sperm send a signal that restarts meiosis. The first polar body (light red) is extruded.



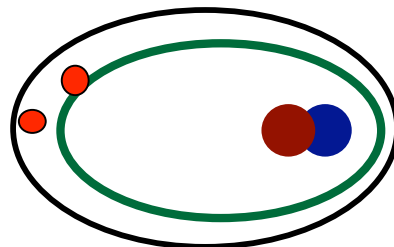
**C:~20'**

PAR6-3 PKC-3 are at the anterior pole (A). At the posterior (P), are sorted PAR 1-2. The second polar body (light red) is extruded.



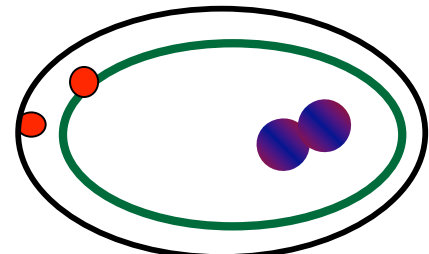
**D:~23'**

The female *pronucleus* (dark red) moves toward the sperm through the pseudocleavage.



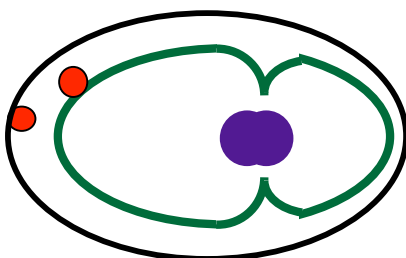
**E:~25'**

The two *pronuclei* meet, their envelopes break-down. They start to mix their haploid DNA contents.



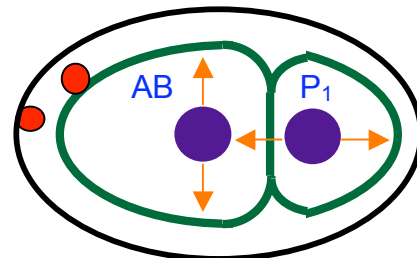
**F:~30'**

The two *pronuclei* move to the center of the embryo by accomplishing a 90° rotation along the AP axes.



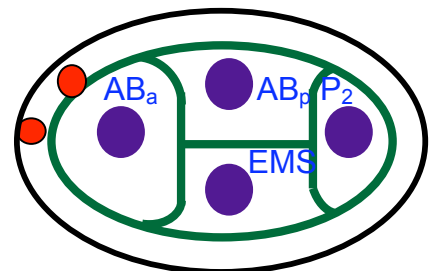
**G:~35'**

Formation of the first mitotic spindle, beginning of the first cleavage.



**H:~40'**

First cleavage: cell-autonomous determinants differentially segregated in the cells **AB** (left) and **P<sub>1</sub>** (right).



**I:~55'**

**AB** cell divide in **AB<sub>a</sub>** and **AB<sub>p</sub>**, followed shortly after by **P<sub>1</sub>**. This cell will divide again, giving rise to **EMS** and **P<sub>2</sub>** cells.

Fig. 1 General view of features characterizing the early stages of the development of *C.elegans*.

The first asymmetric cell division is a fundamental requirement for the normal development of *C. elegans* embryos. Only when specific proteins and granules are restricted in definite regions, the determinants of cellular fates will be correctly sorted and distributed among the blastomeres. In order to achieve an asymmetric first cleavage, the just fertilized monocellular embryo should relocate specific proteins and proceed from the initial symmetric condition present in the oocyte to the polarized state of the fertilized embryo (Sonneville R. and Gönczy P. 2004).

The polarization process involves the sperm centrosome that is required for the initiation of the process (but not for its perduration) (Cowan C.R. and Hyman A.A., 2004a) and it is achieved through the localization of specific proteins in discrete regions of the cell. The markers of polarity are the PAR proteins (PAR stands for abnormal embryonic PARTitioning of cytoplasm) (more details are contained in the “discussion” section of this thesis). These proteins, in the first minutes after fertilization, are distributed throughout the cytoplasm and the cortex in a diffuse manner. However, after the entry of the sperm, PAR proteins are rearranged by movements of the actomyosin network. The cytoskeleton relocates the PAR proteins, some to the anterior and some others to the posterior cortex (Munro E. *et al.*, 2004) (fig.1 C). The first cell cleavage will fall right in between the zones outlined by the two different sets of PAR proteins.

These proteins were discovered by a genetic screen almost twenty years ago (Kemphues K.J. *et al.*, 1988) and almost all are conserved throughout evolution. It has been found that this class of proteins is responsible, in different organisms (*C. elegans*, *Drosophila*, human epithelial cells and neuroblast *etc.*), for the correct establishment of polarity and, indirectly, for the determination of the cell fates (Cowan C.R. and Hyman A.A., 2004b; Knoblich J.A. *et al.*, 2001; Macara I.G. *et al.*, 2004; Onho S. *et al.*, 2001; Schneider S.Q. and Bowerman B., 2003; Wang H. and Chia W., 2005). PAR proteins contain a very well defined protein-protein interaction domain such as PDZ motifs (PAR3-6) or the amino-terminal CRIB domain (that in PAR-6 interacts with Cdc-42 or Rac proteins (Onho S. *et al.*, 2001). Furthermore, the localization and the assembling of PAR protein complexes are modulated by phosphorylations (for a review see in Munro E.M., 2006).

PAR-6 and aPKC associate and phosphorylate PAR-3; thereafter this trimeric complex will localize to the anterior pole of the embryo (the opposite to the sperm entry point). PAR-6, PAR-3 and PKC-3 will actively restrict at the posterior pole the proteins PAR-2 and PAR-1 by a mechanism that has not yet been completely clarified (Etemad-Moghadam S., *et al.* 1995; Watts J.L., *et al.* 1996; Tabuse Y., *et al.* 1998; Hung T.J. and Kemphues K.J., 1999; Cuenca A.A., *et al.* 2003). Another unresolved issue of the polarization process is understanding how the signal that starts the process is sent and how this is perceived by the machinery that puts it in action. A possible connection between the signaling machinery and the polarization process may reside in the discovery that PAR-6 is a downstream effector of the activated form of CDC42, a member of the RHO family small GTPases (Joberty G. *et al.*, 2000). GTP bound CDC42 interacts with the semi-CRIB domain of PAR-6 thus inducing its conformational change. This event constitutes the first step in the building up of the PAR complex at the anterior pole (Garrard S.M. *et al.*, 2003). In response to this polarized state, also the germ line P granules are forced to segregate to the posterior cytoplasm of the embryo at one cell stage (Hird S.N. *et al.*, 1996). This specific sorting ensures that, when the embryo will reach the four cells stage, only the blastomere P2 will receive the P granules (determinants of the germ line) (fig.1 I) (Kawasaki I. *et al.*, 1998).

## 2.5 Synthesis of the eggshell

Simultaneously to the process of cell polarization, another fundamental event in which lipids have been shown to be directly involved occurs. Immediately after fertilization, the rapid synthesis of an eggshell that surrounds the embryo is initiated (fig.1 B). The eggshell is a structure resistant to harsh treatments and protects the embryo even when the mother is bleached by alkaline hypochlorite solution. The eggshell completely isolates the progeny from the mother so that, after its synthesis, no additional interaction between the embryo and the external environment are possible. The oocyte contains enough bio-molecules for the completion of the embryonic development until when, after 14 hours, it hatches L1 larva.

The eggshell of nematodes is a structure with an average thickness of 200 nm (although it is wider at the poles) and is composed of three different layers (fig.16 and 20). The most external part of a wild type eggshell is a thin layer derived from the oolemma, called **vitelline layer**. Although its composition has not yet been clarified, lectin-labeling techniques have detected carbohydrate residues on the surface of the eggs of several nematodes (Bird A.F. and Bird J.,1991).

The **chitinous layer** is the second and most prominent sheet of the eggshell. It provides the mechanical support and gives the embryo its typical ellipsoid shape (wild type embryos whose chitin layer has been removed tend to round up and become very sensitive to mechanical stress.) (Schierenberg E. and Junkersdorf B., 1992; Zhang Y. *et al.*, 2005) The innermost **lipid-rich layer** acts as a solvent barrier; its composition in the nematode *Ascaris* is supposed to be made by lipids (75%) and proteins (25%) (Fairbairn D., 1957).

Unfortunately very little is known at the moment about the chemical composition of the three layers and about the biosynthetic pathway that leads to the production and assembly of the eggshell. Understanding how this protective structure is generated would have a remarkable importance because this envelope shields also the species of parasitic worms that are dangerous for humans during the most delicate step of their life cycle: the embryonic development. The identification and characterization of enzymes involved in the eggshell synthesis would be very important because they would lend themselves as excellent candidates for designing drugs against parasite nematodes. Until few years ago only the description of the three layers of the eggshell by EM (Electron Microscopy) was known (Bird A.F. and Bird J.,1991; Rappleye C. A. *et al.*, 1999). Recently, however, some data that might start to clarify the possible composition of the eggshell were reported. CHS-1 (CHitins Synthase), for instance, has been identified as the enzyme that polymerizes the polysaccharide N-acetyl-D-glucos-2-amine and thus produces chitin for the central layer (Zhang Y. *et al.*, 2005). Even fewer data are available for the other two layers. Only one mutation has been so far described to alter the eggshell structure. This mutant is *Pod-1(ye11)* (POD stands for Polarity and Osmotic sensitivity Defect); EM observations of the eggshell of this mutant revealed that the innermost layer is duplicated

(Rappleye C. A. *et al.*, 1999). *Pod-1* encodes for a coronin-like protein and therefore it is a member of a class of protein that regulates the actin cytoskeleton and governs vesicular trafficking (Rybakin V. and Clemen C.S., 2005). The replication of the lipid-rich layer and the consequent non functional eggshell is likely to be caused by impaired exocytosis of the material constituting the eggshell, or by failure in endocytosis of the overexceeding material (Rappleye C. A. *et al.*, 1999). Even if this mutant did not give information about the possible chemical composition of the inner layer, the description that a defective eggshell results in a POD phenotype has permitted the screening and identification of other genes with similar phenotypes and evaluate their potential implication in eggshell formation. One of the mutants identified in this screen is *pod-2(ye60)* that codes for an Acetyl-CoA carboxylase (Tagawa A. *et al.*, 2001; Rappleye C. A. *et al.*, 2003). Acetyl-CoA carboxylase performs the first step in long chain fatty acid synthesis and this evidence suggest the possibility that FAs (fatty acids) may be involved in the creation of the eggshell thus being essential for the development of *C. elegans* embryos.

## 2.6 Cytochrome P450s

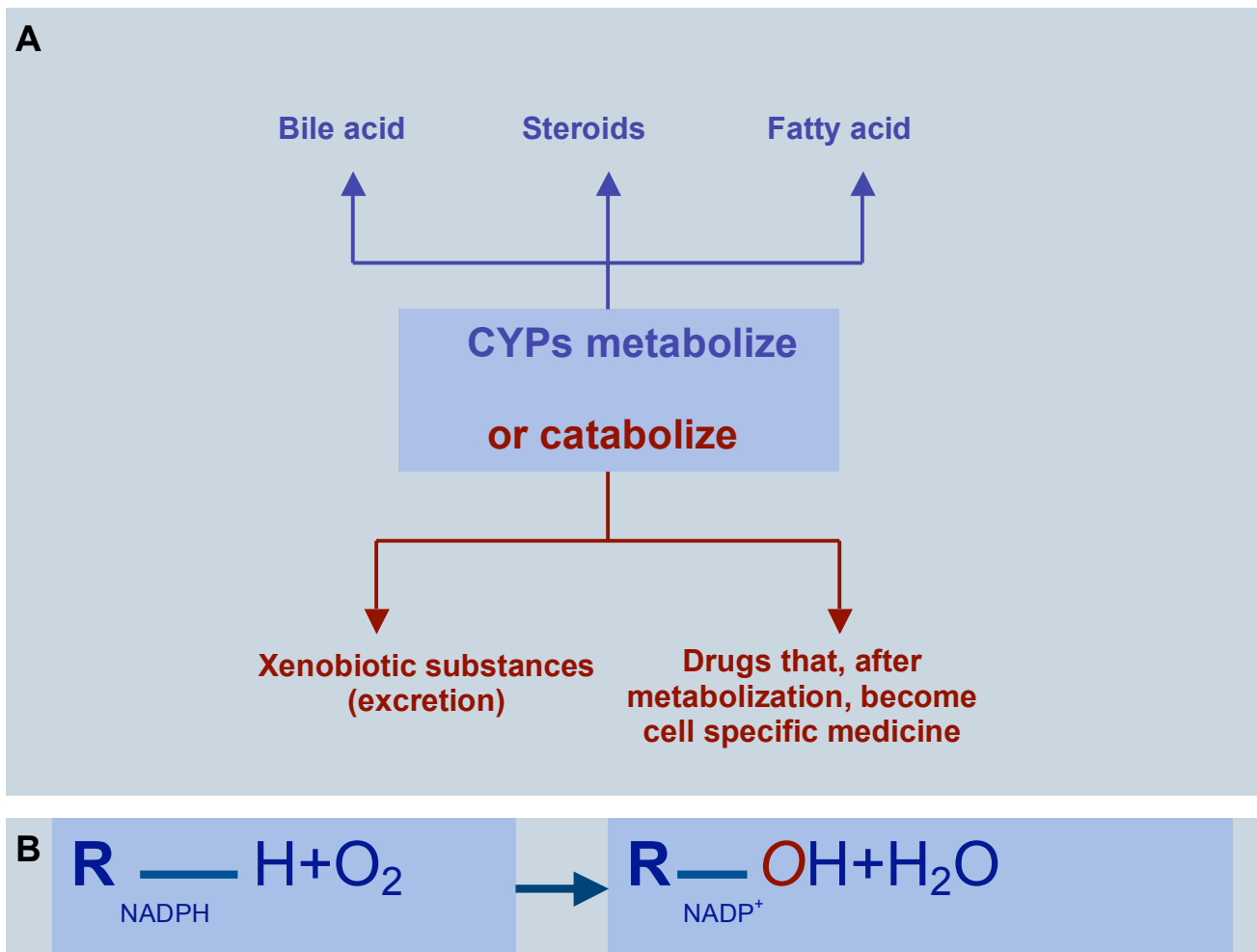
In our laboratory, we aim to characterize the pathways based upon lipid intermediates that are involved in the regulation/activation of specific steps development of *C. elegans*. For instance, the role of cholesterol and cholesterol-derived molecules and their capability to regulate the entry and the exit from the “dauer” larvae developmental stage has been investigated (Matyash V. *et al.*, 2004). More recently, the research field has been extended to other classes of lipids like phospholipids and FAs. (Dominik Schwudke *et al.*, 2006; Entchev E. *et al.* submitted, 2006).

We have focused our investigation on the identification of enzymes that can modify lipids. One class of such enzymes is the cytochrome p450 protein family. Cytochrome P450 is one of the largest superfamilies of enzymes and it takes its name by the characteristic absorption band of the heme (called the Soret peak) at 450 nm. (Klingenberg M., 1958; Omura T. and Sato R., 1964)

P450s are heme-thiolate proteins (heme is a chemical structure that consists of an iron atom contained in the center of a large heterocyclic organic ring); their most conserved structural features are related to heme binding, the major feature being a completely conserved cysteine serving as a ligand to the heme iron (Werck-Reichhart D. and Feyereisen R., 2000; see also fig. 5). Bioinformatics analysis of the *C. elegans* genome revealed 80 members of the cytochrome P450s (Nelson D.R., 1999; revised to 83 in March 2004 (<http://drnelson.utmem.edu/revised.Celegans.seqs.htm>) (in humans there are about 55 genes and 25 pseudogenes) (Werck-Reichhart D. and Feyereisen R., 2000). Intriguingly, with an exception for the CYP DAF-9 that is involved in dauer formation (DAF stays for DAuer Formation) (Jia K. *et al.*, 2002; Gerisch B. and Antebi A., 2004; Gerisch B. *et al.*, 2004), almost nothing is known about the functions of other CYPs in *C. elegans*.

The functions of the CYPs are extremely diverse; cytochrome P450 can be found in all types of tissues, with developmentally regulated patterns of expression. The reactions carried out by P450s are mostly hydroxylation or epoxidation. Canonical CYPs use electrons from NAD(P)H to catalyze activation of molecular oxygen, leading to regiospecific and stereospecific oxidative attack of several substrates (fig.2). They catalyze regiospecific and stereospecific oxidative attack of non-activated hydrocarbons at physiological temperatures. The details of the mechanism by which P450s carry out all types of reactions are not yet understood. CYPs contribute to vital processes such as carbon source assimilation, biosynthesis of hormones and structural compounds in general, and detoxification from xenobiotics substances. The P450 genes are found in genomes of virtually all organisms. In eukaryotes, they are usually bound to the endoplasmic reticulum or inner mitochondrial membranes however, there is no established rule to predict the sub-cellular distribution of P450 enzymes. These enzymes are integral membrane proteins (Ekstrom G. and Ingelman-Sundberg M., 1984) and are involved in both synthetic and degradative reactions of thousands of endogenous and exogenous compounds such as vitamins, steroids, fatty acids and prostaglandins but they are also implicated in the detoxification of many xenobiotic substances (fig.2).

Fig.2



**Fig. 2 Metabolism of cytochrome P450s**

Cytochrome P450 are involved in the synthesis and degradation of several classes of compounds (**A**). The typical metabolic reaction of a Cytochrome P450 (**B**).

P450 superfamily genes are subdivided and classified following recommendations of a nomenclature committee, on the basis of amino-acid identity, phylogenetic criteria and gene organization. The root symbol CYP is followed by a number for families (generally groups of proteins with more than 40% amino-acid sequence identity, of which there are over 200), a letter for subfamilies (greater than 55% identity) and a number for the gene; for example, CYP-44-A-1 (Nelson D.R., 2006; <http://drnelson.utmem.edu/CytochromeP450.html>).

The diversity in the cytochrome P450 superfamily has risen by an extensive process of gene duplication and by gene amplifications,

conversions, genome duplications, gene loss and lateral transfers. “Fossil” evidence of these processes can be found by careful sequence alignments. Worms lack mitochondrial cytochrome P450s. The mitochondria are the organelles in which, in mammals and in insects, the synthesis of steroid hormones takes place (Nelson D.R., 1998). This deficiency may give the impression of a very different lipid metabolism, however, *C. elegans* presents an elevated number of cytochrome P450s. Moreover, the degree of conservation among the protein sequences suggests that the lipid metabolic products can potentially be similar between nematode and mammals. Several clinical disorders have been related to mutations in CYP genes (Okita R.T. and Okita J.R., 2001). Additionally, their clinical relevance is enhanced by the fact that they can catabolize several prodrugs commonly used as cancer chemotherapeutics.

## **2.7 Identification of small metabolites in *C. elegans***

Functional genomic or proteomic data are not adequate to give unambiguous information on the metabolic product(s), activity and function of many annotated enzymes. In a genetic system such as *C. elegans*, identification of enzymes and relevant products can be done by: I) selecting mutants that fail to synthesize metabolites already known; or II) by the reverse approach, where metabolites are identified based on their capability to complement mutants of a particular gene. A successful example of the first approach is the dissection of the synthesis of PolyUnsaturated Fatty Acids (PUFA) in *C. elegans* (Watts J.L. and Browse J., 2002) where a set of mutants was isolated on the basis of their abnormal FA composition.

The reverse complementation approach consists of the identification of fractions of the total lipids that rescue phenotypes caused by absence of protein function (mutants or RNAi) (Watts J.L. and Browse J., 2006; Rappleye C. A. *et al.*, 2003; Motola D.L. *et al.*, 2006). The discovery of dafachronic acid represents a success case of this approach. This acid was identified as the metabolic product of the cytochrome P450 DAF-9 because it was able to restore the reproductive development in dauer constitutive *daf-2* and *daf-9* mutants (Motola D.L. *et al.*, 2006). An additional case is represented by the



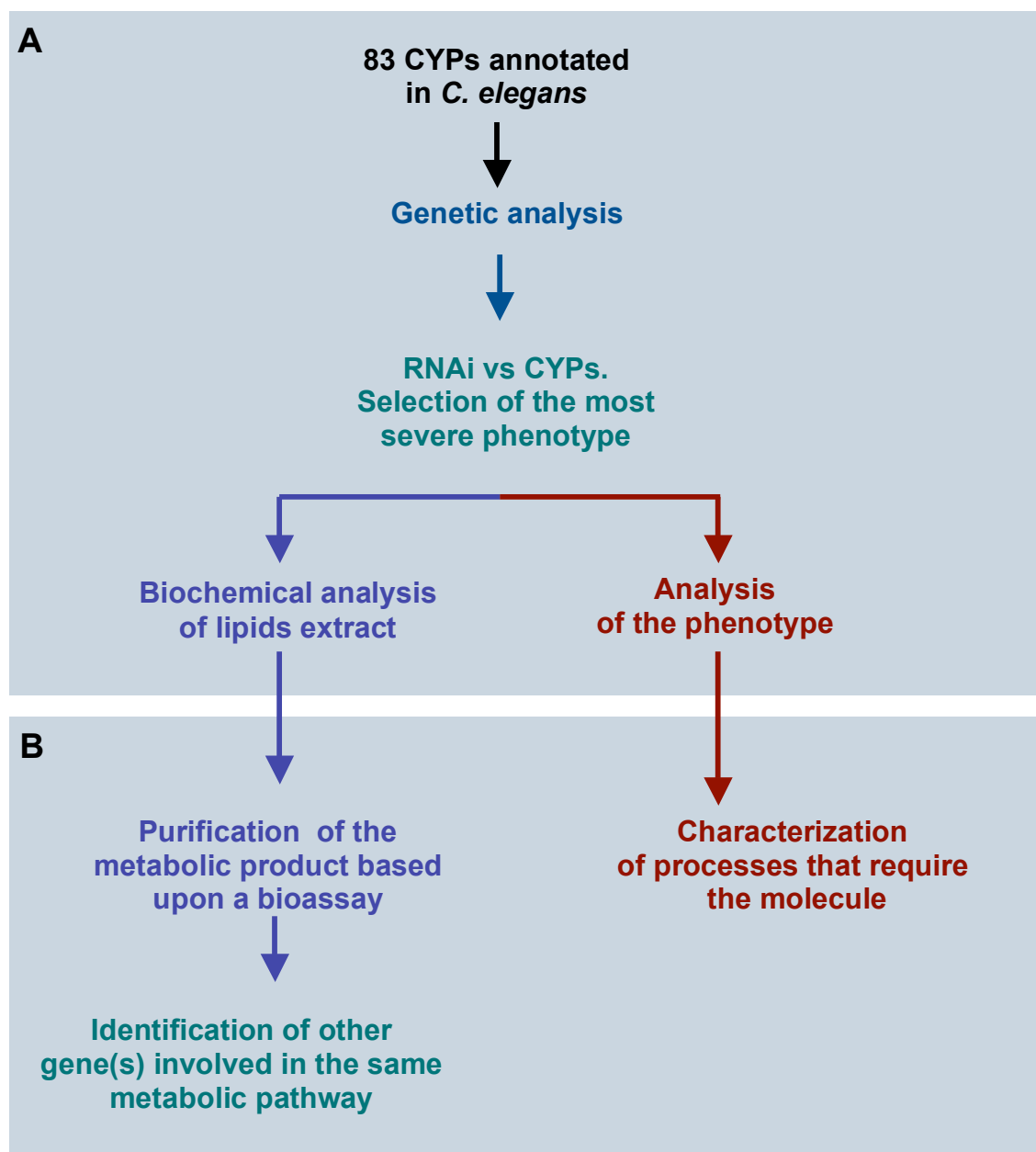
capability of FAs to rescue polarity defects when supplemented to the already described *pod-2(ye60)* mutants (Rappleye C. A. *et al.*, 1999). The complementation approach is very appealing because in addition to mutants, it can be also applied to RNAi treated worms.

The capability of the worms to adsorb basal lipids from the growth media can be demonstrated by the uptake of radioactively labeled acetate or cholesterol. These lipids are further metabolized to the respective metabolic products (fig. 18). An explanation for such an ability is the fact that *C. elegans* is a soil organism, and because it is a crawler the worm is constantly in direct contact with the environment. This characteristic has probably affected its evolution in such a way that the worm has lost its capability to synthesize molecules that are easy to find in the surroundings. Thus, the synthesis of the relative enzymes needed for the production of these molecules would be an unfavorable dispense of energy. On the other hand, *C. elegans* retains a great capability to take up precursors such as sterols of yeast, plant or mammalian origin, and through their modifications create the great number of derivatives that its metabolism requires. If, for instance, we analyze the case of cholesterol: mammalian cells can either uptake this molecule from the media or can synthesize it starting from acetate. In mammals both pathways can be needed because cholesterol is massively used (it typically constitutes 20% of the total lipid content of animal cell plasma membranes) (Haines T.H., 2001). On the contrary, the nematode *C. elegans* has been found to need a very small amount of this molecule (2.5 ng ml<sup>-1</sup> cholesterol for 1 h are sufficient to rescue the effect of cholesterol depletion in arrested larvae) (Kurzchalia T.V. and Ward S., 2003). The genes coding for the proteins for the *de novo* synthetic pathway (that synthesize cholesterol starting from acetate) have not been found in the *C. elegans* genome (<http://www.genome.ad.jp/kegg/kegg2.html>). The abundance of sterols available in the media, coupled with the capability to uptake them in different forms, are indeed enough to guarantee the worm a normal development.

## AIM OF THE THESIS

The purpose of this thesis is to identify lipid(s) essential for the development of *C. elegans* and enzyme involved in the synthesis of such

Fig.3



**Fig. 3 Strategy and aim of the thesis**

Representation of the experimental strategy of the thesis (A).

The targets that this thesis is aiming to fulfill (B).

lipids. An extensive bioinformatics analysis led us to focus on a member of the CYP family involved in a crucial biosynthetic metabolic pathway.

When this CYP was silenced by feeding with dsRNA producing bacteria, the worms displayed embryonic lethality. The defects that led to the arrest of the development were characterized in more detail by means of light and electron microscopy techniques. The description of the phenotype permitted us to discover that the metabolic product of the CYP that we are investigating is required to build a functional eggshell. A defective eggshell is associated with the osmotic sensitivity, failure in establishment of polarization and in the polar body extrusion.

Simultaneously, we started a purification procedure in order to isolate from the lipid extract the metabolic product of the CYP450. In order to test and identify the fractions of lipid extract that are able to rescue the embryonic lethality observed after RNAi, we set up a bioassay.

**Conclusively, the result of this thesis consisted in the investigation of a cytochrome P450 and its metabolic product, essentially required for the development of *C. elegans* embryos. (fig.3).**

### 3- RESULTS

#### 3.1 Bioinformatic analysis of enzymes involved in lipid synthesis

With the intention of investigating developmental mechanisms regulated by lipids, we planned a RNAi approach to silence specific CYPs and CYP-related proteins. In order to select genes that are possible producers or modifiers of essential lipids, an extensive bioinformatic analysis was carried out. The aim of the analysis was to focus our project on proteins involved in a synthetic pathway required for development. By this study, we wanted to avoid CYPs that perform catabolic reactions (thus, are mainly involved in the detoxification of the organism). The results of this study are presented in table 1. These proteins were selected because they fulfilled several criteria: homology with enzymes characterized as steroid hormone producers in other organisms, involvement in an already characterized lipid metabolic pathway, specific cellular localization, expression pattern based on microarray data, *etc.* (table 1).

Table n.1

<u>Genes</u>	<u>ORF</u>	<u>Function</u>	<u>Phenotype observed</u>
1- <b>PBR</b>	C41G7.3	Mitochondrial transporter of cholesterol	WT
2- <b>CYP44A1</b>	ZK177.5	High Homology with <i>Disembodied</i> CYP that produces Ecdysones in <i>Drosophila</i>	WT
3- <b>Adrenodoxin</b>	Y73F8A.27	Electron transfer to CYP	WT
4- <b>Adrenodoxin reductase</b>	Y62E10A.6	Electron transfer to CYP	L3 stage arrest
5- <b>CYP31A2</b>	H02I12.8	CYP with unknown function	Emb

In order to silence these proteins we performed RNAi by feeding: part of the genes of interest (0,5 - 1,5 Kbp) were amplified by PCR (Polymerase Chain Reaction) from *C. elegans* genomic DNA and cloned into a vector for the expression of dsRNA (Timmons L., Fire A. 1998; Timmons L. *et al.*, 2001). The worms were fed with bacteria producing dsRNA and the resulting morphological phenotypes were monitored and annotated (table n.1). When *C. elegans* were fed with *cyp31A2(RNAi)* bacteria, the silencing of the gene caused a remarkable developmental effect: *emb* (EMBryonic lethality). We decided to focus our project on studying the function of the protein CYP31A2.

### **3.2 Characterization and phylogenetic analysis of the gene *cyp31A2***

Several characteristics rendered the gene *cyp31A2* valuable for our research and numerous reasons convinced us to investigate the metabolic reaction carried out by this enzyme:

- The amino acid sequence analyzed using BLAST (Basic Local Alignment Search Tool ver 2.1) (Altschul S.F. *et al.*; 1990; 1997) revealed high homology with the family of cytochrome P450 number 4. The homology with the family 4 was interesting for us because cytochrome members of this family are involved in the modification of sterols and fatty acids (FA) (fig. 4).

These two classes of molecules are considered to be important modulators of many cellular processes. While steroid hormones are well established as signaling molecules (Beckstead R.B. and Thummel C.S. 2006), the importance of fatty acids in the transduction of signals has recently emerged. For instance, germline proliferation in *C. elegans* begins in the late L1 larval stage and continues throughout adulthood (Seydoux G. and Schedl T., 2001). Dihomomogamma-linolenic acid (DGLA) (a PUFA) dietary supplementation during larval development results in sterile adults without germ cells. This observation could be explained by signals that inhibit cell proliferation or the activation of degenerative signals in the germ cells (Watts J.L. and Browse J., 2006).

- In spite of the importance of cytochrome P450s, in the genome wide screens performed by RNAi in *C. elegans*, significantly strong phenotypes

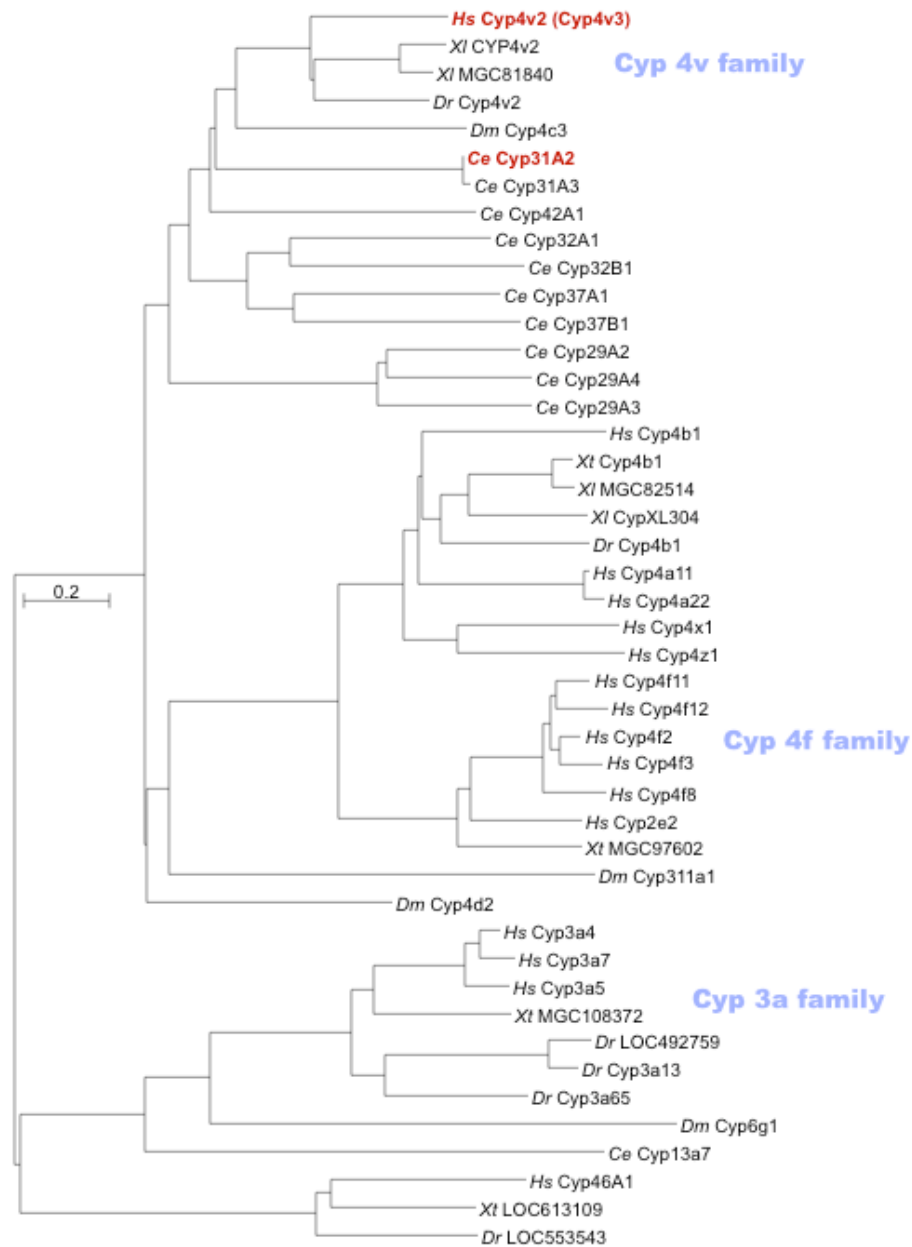
have been reported only for few of them (<http://drnelson.utmem.edu/C.elegans.RNAi.html>).

Piano and co-workers carried out a screen by microinjection of dsRNA against genes expressed in the ovary. In this screen they also included *cyp31A2* and the corresponding reported phenotype was embryonic lethality (Piano F. *et al.*, 2002).

-The protein sequence of CYP31A2 is conserved through evolution and, in a BLAST comparison the amino acidic sequence shows 65% of similarity (45% identity) with the human cytochrome CYP4V2 (in red in fig. 4). The human gene *cyp4v2* has recently been linked by genetic analysis to a severe pathology called “Bietti Crystalline corneoretinal Dystrophy” (BCD) (Jiao X. *et al.*, 2000; Lee J. *et al.*, 2001; Li J. *et al.*, 2004; Lee K.Y. *et al.*, 2005; Lin J. *et al.*, 2005; Shan M. *et al.*, 2005). The connection to the BCD pathology offered an additional clinically-linked reason to investigate the *C. elegans* protein CYP31A2. The high percentage of conservation of the proteins between human and nematode CYPs led us to speculate that the characterization of *C. elegans* CYP31A2 might provide information about the human protein and the disease that it causes.

Fig.4

### Phylogenetic Tree of the Cytochrome P450 Family



**Fig. 4 Phylogenetic tree of the cytochrome P450's family**

The diagram shows the evolutionary distances among protein sequences from different organisms.

In red, are indicated *C. elegans* CYP31A2, the human (CYP4V2) and the murine (CYP4V3) homologues.

**Hs:** *Homo sapiens*, **Xl:** *Xenopus laevis*, **Dr:** *Danio rerio*, **Dm:** *Drosophila melanogaster*, **Ce:** *Caenorhabditis elegans* **Xt:** *Xenopus tropicalis*.

**cyp31A2** (ORF H02I12.8) gene spans 2036 bp of DNA sequence. The six exons generate a transcript of 1448 bp and a protein 495 amino acids long: the gene model can be seen at <http://wormbase.org/db/gene/gene?name=h02i12.8>).

Analysis of the sequence of the protein CYP31A2 revealed the presence of several conserved and characteristic domains:

- The CYP signature motif FxxGxxx**C**xG (Feyereisen R., 1999) is present between the residues 445–454 in fig.5. Within these amino acids, the cysteine residue (in bold) is responsible for the binding with the heme iron present in mature CYP enzymes (Gotoh O., 1983).

- Residues 308–320 in fig. 5 (EVDTFMFEGHDTT) are a sequence motif characteristic of all the family 4 CYPs (CYP4V2 included) (Bradfield J.Y. *et al.*, 1991) (fig. 5);

- The presence of PxxFxP (residues 421–427 in fig.5) indicates a possible microsomal localization (i.e., CYP31A2 probably binds to the membrane of the endoplasmic reticulum rather than to mitochondria) (Graham-Lorence S. and Peterson J.A., 1996).

- The helix by which CYP31A2 can be inserted in the microsomal membrane is enclosed in the first 21 amino acids (fig. 5).

In order to identify proteins with sequences similar to CYP31A2 in other organisms and analyze their functions, we compared and aligned their amino acid sequences. The results of this bioinformatics analysis produced the phylogenetic tree depicted in fig. 4.



Fig.5

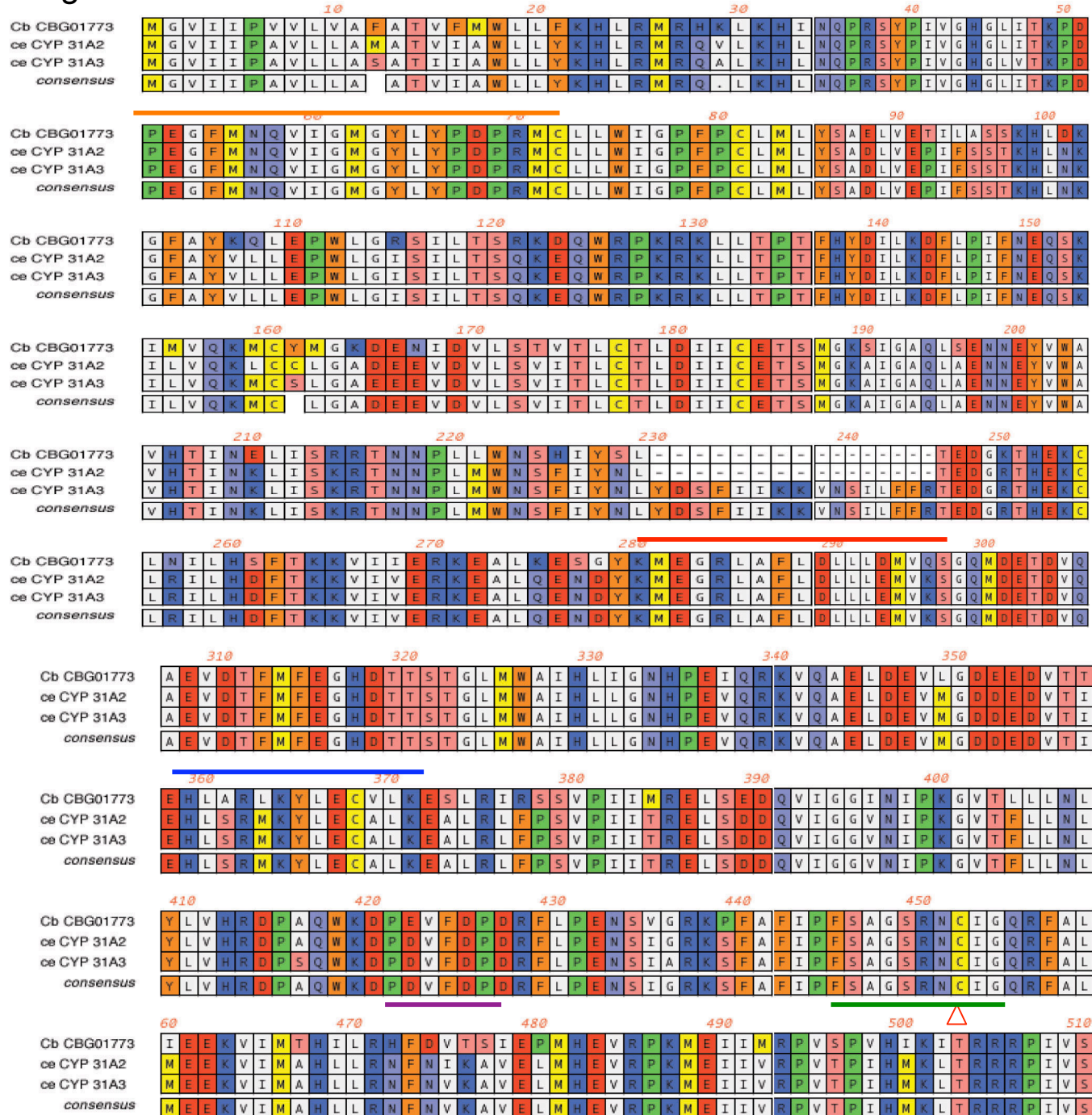


Fig. 5 Aligning of the CYP31A sequences

From the top: *Cb* CBG01773 (*C. Briggsae* homologue), *Ce* CYP31A2, *Ce* CYP31A3 and the consensus of the three CYPs.

Note the extremely high level of conservation among the three proteins.

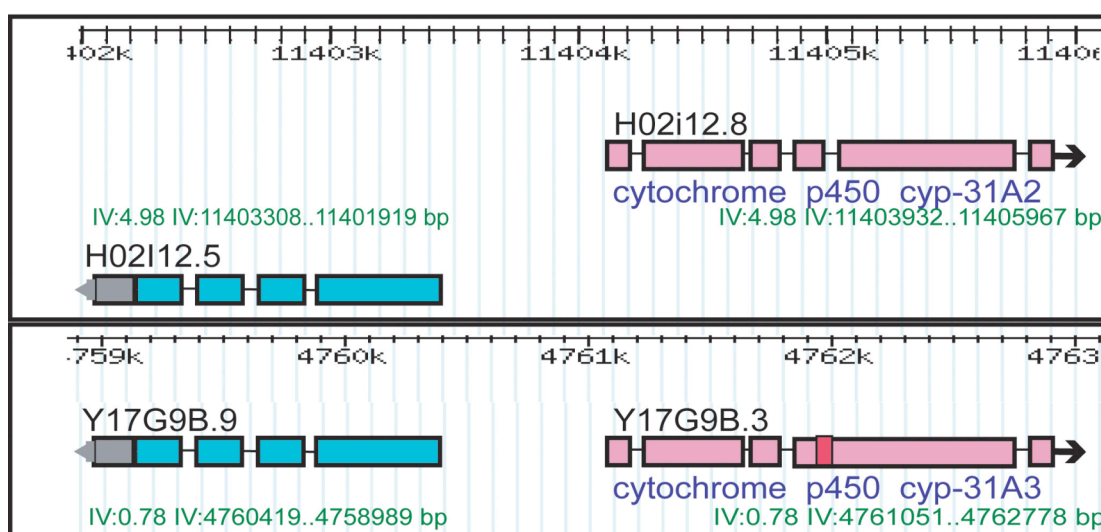
- Extra region
- Family 4 signature motif
- Heme binding motif
- △ Cysteine binding heme's group
- Microsomal localization
- Membrane insertion sequence

The comparison of the sequences detected the presence of a *C. elegans* protein sequence with a high degree of similarity to our target protein: the cytochrome P450 CYP31A3 (fig. 4, 5 and 6). The two primary sequences shared 96 % of amino acids similarity where 94% were identical. The main differences were detected in an extra sequence that coded for the 16 amino acids in position 230-246. The nucleotide sequence that coded for this “extra region” in *cyp31A3* was annotated as a coding sequence that links the exon number four and five into a bigger fourth exon (in red in fig. 6). The genomic locus of *cyp31A2* contained this sequence too, but it was annotated as an untranslated intron (fig. 5 and 6).

The 16 amino acids in this extra region did not code for any obvious or already characterized domain and we were not able to make a hypothesis about its possible function. When the genomic sequences of *cyp31A2* and *cyp31A3* were compared with the genome of *C. briggsae*, the search found a single homologous to both genes in *CBG01773*. Although mistakes are possible in the gene assignment of *C. briggsae* genome project, the data collected so far permitted us to hypothesize that the two *C. elegans* genes *cyp31A2* and *cyp31A3* might have been generated by duplication of the common *C. briggsae*'s ancestor, *CBG01773*. Furthermore, the comparison of the three amino acidic sequences suggested that CYP31A2 could be the more ancient protein because the extra sequence (230-246) is neither present in CYP31A2, nor in the possible ancestor *C. briggsae* *CBG01773* (fig. 5). The cytochrome P450 proteins are known to have arisen by gene duplication. CYPs diverged from the ancestors by the gain and loss of multiple introns. This class of proteins evolved with an extremely high variability and this divergence is reflected in the wide spectra of metabolic reactions that they perform nowadays. The process that led to the divergence of sequences in order to create new genes was so effective that the general sequence identities among P450 proteins of different classes is extremely low (around 20%). (Graham S.E. and Peterson J.A. 1999; Gotoh O. 1998). In contrast, the comparison of CYP31A2 and CYP31A3 protein sequences reported 96% of similarity (fig. 6). The homology was conserved also at the nucleotide level: *cyp31A2* and *cyp31A3* gene sequences share 91% similarity. The two genes were mapped to the same chromosome (the fourth) but in different *loci* (fig. 6).

The analysis of the two loci detected sequence homologies in the putative promoter regions and in the genes that precede *cyp31A2* and *cyp31A3* in the map too (fig. n.6). A third member of the family (the gene *cyp31A1*) has been classified as an untranscribed pseudogene thus it has not been annotated in the phylogenetic tree in fig. 4 (<http://wormbase.org/db/gene/gene?name=c01f6.3>).

Fig.6



**Fig. 6 Genomic organization of the loci *cyp31A2* and *cyp31A3***

*cyp31A2* and *cyp31A3* are located on the same chromosome (IV) and they have almost identical genomic organization. The “extra region” is, in the scheme, marked in red.

The sequence of this region is predicted to code for an intron in *cyp31A2*, whereas in *cyp31A3* it is included in the fourth exon.

The similarity between the two sequences is conserved also in the promoter regions and in the genes that precede *cyp31A2* and *cyp31A3* in the genetic map.

The scheme of the loci are taken and modified from [www.wormbase.org](http://www.wormbase.org)

*cyp31A2* and *cyp31A3* have sequences too similar to be specifically targeted by dsRNA ([http://nematoda.bio.nyu.edu/cgi-bin/rnai/browse/search\\_results.rnai.cgi](http://nematoda.bio.nyu.edu/cgi-bin/rnai/browse/search_results.rnai.cgi)). The impossibility to discriminate between *cyp31A2* and *cyp31A3* genes by RNAi led us to treat these two transcripts as a single gene. In agreement with this, in the genome wide-screens that included these two genes, they have been always annotated as possible off-targets for each other. Thus, *cyp31A* will be referred in this thesis as the combination of the two RNAi targets for *cyp31A2* and *cyp31A3*.

### **3.3 *cyp31A(RNAi)* leads to embryonic lethality**

When *C. elegans* were fed with bacteria producing *cyp31A* dsRNA, animals of the first generation were identical to control worms in morphological appearance and developmental timing (data not shown). In contrast, the eggs laid by the *cyp31A(RNAi)* and control worms revealed remarkable differences. After 24 hours, among the eggs that were laid by control worms, 98.2% of the embryos had produced normal L1 larvae (L1 stays for Larval stage 1). At the same time, in the progeny of worms exposed to RNAi, only 8.9% of the laid eggs produced L1 larvae whereas 91.2 % stopped during the embryonic development (fig. 7).

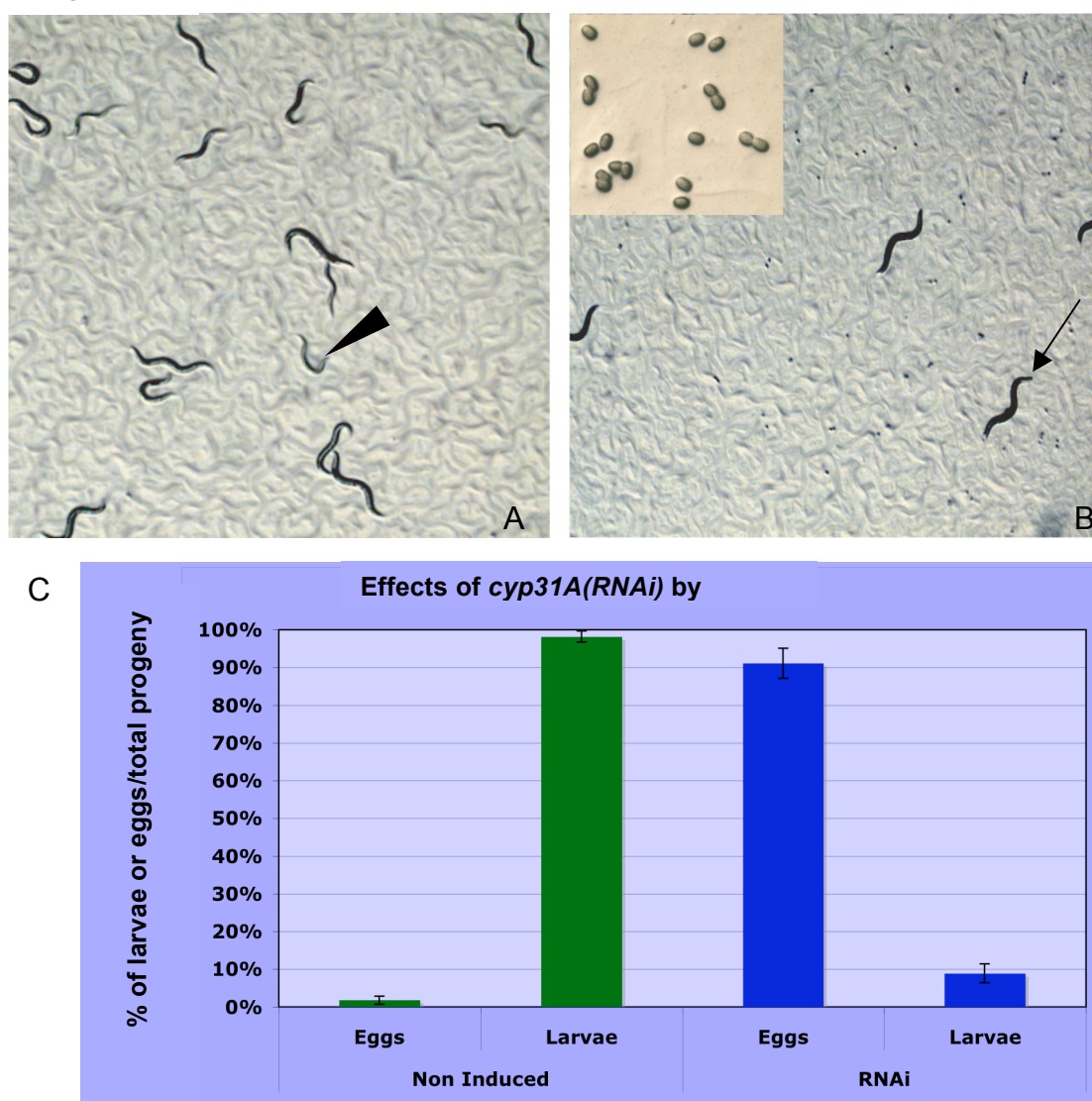
The difference of penetrance between the embryonic lethality detected in our RNAi experiments and those reported by Piano and co-workers (91% vs. 100%) is probably caused by the different methods with which the dsRNA was delivered to the worms (feeding vs. microinjection, respectively).

Because we aimed to carry out biochemical experiments that require a large quantity of RNAi worms, we decided to use the feeding method for “large scale experiments” whereas microinjection is better suited to experiments that require the analysis of few sample worms.

### **3.4 Analysis of the arrest induced by *cyp31A(RNAi)***

In order to address at which stage of the development the *cyp31A(RNAi)* eggs were arrested, Differential Interference Contrast (DIC)-microscopy images were acquired. The images revealed that embryogenesis was arrested at approximately the same developmental stage in all the embryos and no significant difference in the number of cells generated was observed. The embryos stopped their development before reaching the “comma” stage: this stage is so named because worms morphologically resemble the shape of a “comma”. In contrast, the arrested embryos laid by *cyp31A(RNAi)* worms looked like an unshaped cluster of cells (fig. 7 B upper square).

Fig.7



**Fig. 7 *cyp31A*(RNAi) causes embryonic lethality**

RNAi against *cyp31A* does not affect the first generation and therefore the adult worms have a normal appearance (**arrow in panel B**).

Eggs laid from control worms produce normal *larvae* (**Panel A-arrowhead**).

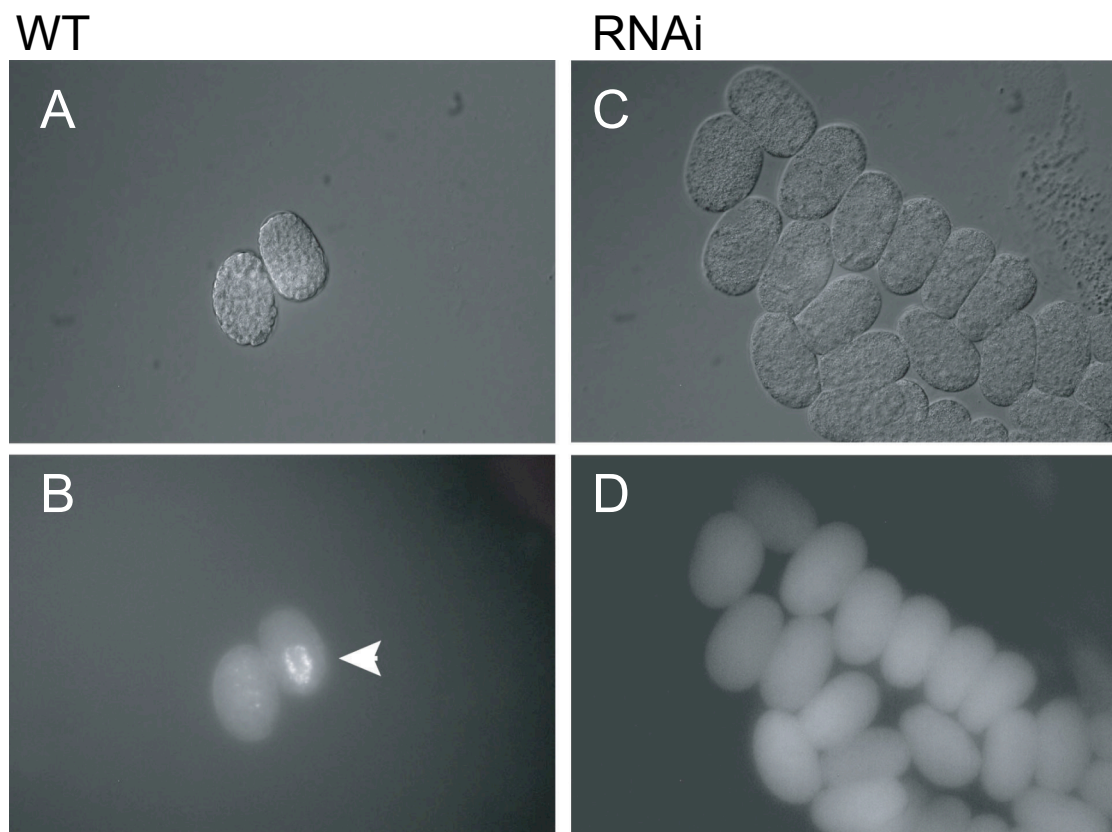
Eggs laid by *cyp31A*(RNAi) worms display embryonic lethality (**Panel B**). The same embryos are shown at bigger magnification in the **insert (B)**.

Quantification of the RNAi effects: the percentage of *larvae* or undeveloped eggs/ total amount of laid eggs is presented in the graphic (**Panel C**).



Another method to determine at which stage *cyp31A(RNAi)* embryos stopped their development was to observe the eggs using fluorescence microscopy. Normal embryos show accumulation of autofluorescent granules during gastrulation that render the 28 intestinal precursor cells visible (for this reason these organelles are called “gut granules”) (Siddiqui S.S. and Babu P., 1980). These birefringent granules have recently been proven to be lysosome-related organelles that contain lipofuscins (fluorescent lipids) (Hermann G.J. *et al.*, 2005; Clokey G.V. and Jacobson L.A. 1986). We could detect the presence of gut granules in wild type embryos whereas they were not observed in images from *cyp-31A(RNAi)* embryos (fig. 8). This observation suggested that the arrest in *cyp-31A(RNAi)* embryos must occur before the end of gastrulation (when the intestinal cell fate is specified).

**Fig.8**



**Fig. 8 *cyp31A(RNAi)* embryos arrest their development before gastrulation**

DIC images of wild type eggs DIC (A).

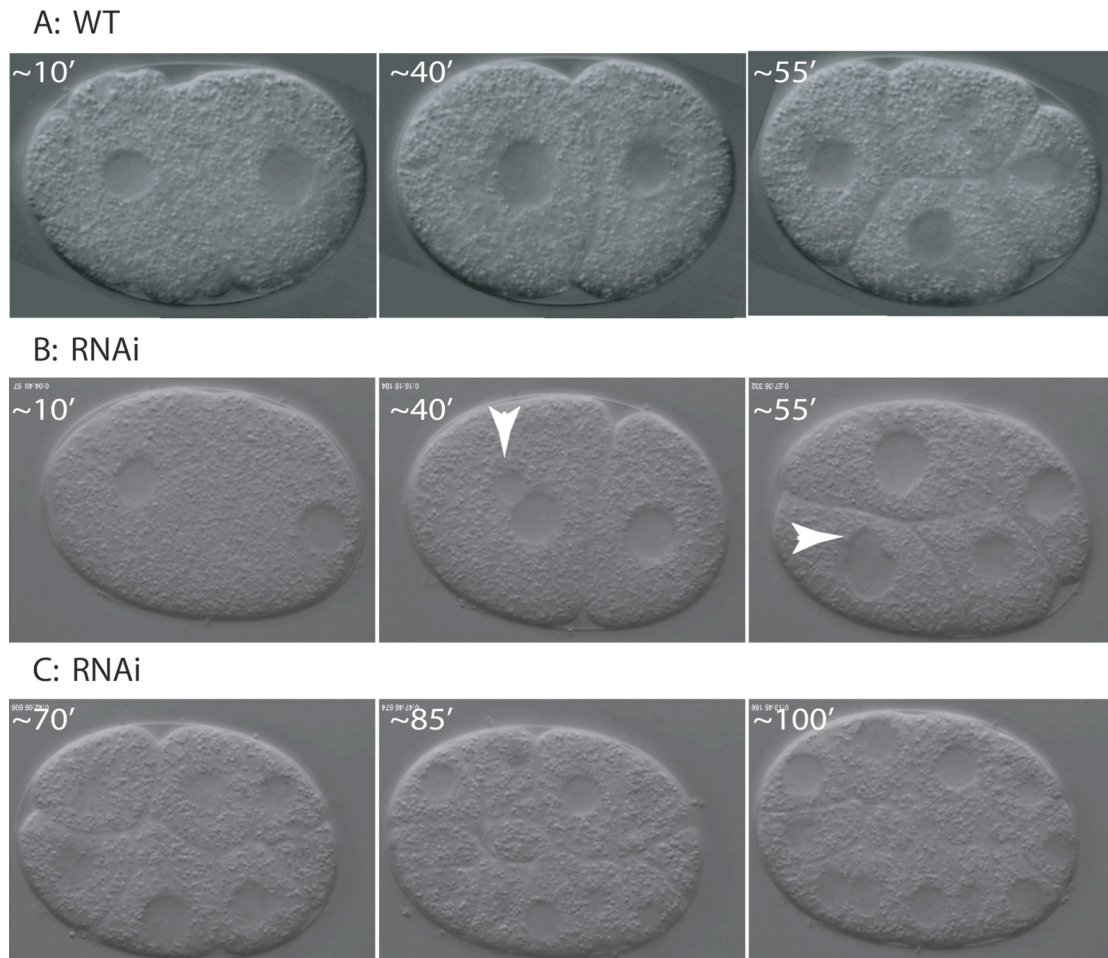
The birefringent gut granules in wild type embryos are indicated by **arrowhead** (B).

DIC images of *cyp31A(RNAi)* embryos (C).

*Cyp31A(RNAi)* embryos (D).

With the intention of narrowing down the exact stage at which the development of the embryos was arrested we took time lapses DIC images.

Fig.9



**Fig.9 DIC-time lapses show that *cyp31A(RNAi)* embryos display problems since the beginning of the development and eventually arrest after ~100'.**

Single frames (the time points are indicated in the frame of the pictures) of time lapses with development of a wild type embryo (**A**) and a *cyp31A(RNAi)* (**B**).

The polar body that is re-inserted in the cell is indicated by **Arrowheads in B**.

Although *cyp31A(RNAi)* embryos show defects since the beginning of the development, after ~100 minutes they stop to proliferate and die (**C**).

This set of DIC time lapses revealed that the embryos stopped to divide approximately 100 minutes after fertilization, that is at the beginning of gastrulation (fig. 9).

### **3.5 *cyp31A(RNAi)* embryos fail to extrude a polar body**

Although *cyp31A(RNAi)* embryos were able to produce blastomeres until the developmental stage of gastrulation, DIC-time lapses detected remarkable irregularities already in the processes that took place just after fertilization.

-The extrusion of the two polar bodies was rarely detected.

The failure in the polar body extrusion results in an extra set of chromosomes in the cell. The polar body that was internalized followed the female pronucleus during its migration and eventually fused with the just formed zygotic (diploid) nucleus.

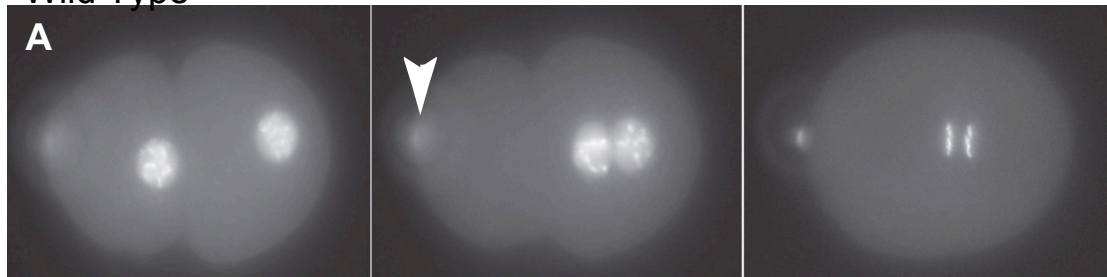
In order to confirm the data observed by DIC, additional movies with a histone H2B::GFP transgenic line were recorded (fig. 10). This strain offered us the possibility to follow the movements of the DNA to which the fluorescent nucleosomes are bound and to track pronuclei and nuclei during their movements (fig. 1). These movies confirmed that the polar body was not properly extruded but was reinserted in the cell, creating embryos with an altered ploidy (fig. 10 A and B).

To investigate if a more severe protein depletion could lead to more penetrant defects, L4 (Larval stage 4) hermaphrodites or young adults were microinjected with dsRNA of *cyp31A*. The embryos in which CYP31A was silenced by microinjection, arrested at an earlier stage of development. The initiation of the meiotic process was observed and the chromosomes were duplicated. In normal conditions the meiotic process is accomplished in the first thirty minutes after fertilization. However, microinjected *cyp31A(RNAi)* embryos were unable to resolve the chromosomes after forty-fives minutes. After this period of time, the movements of the cytoskeleton were observed to slowed down and eventually the development was arrested before accomplishing the first mitotic division (fig. 10 C).

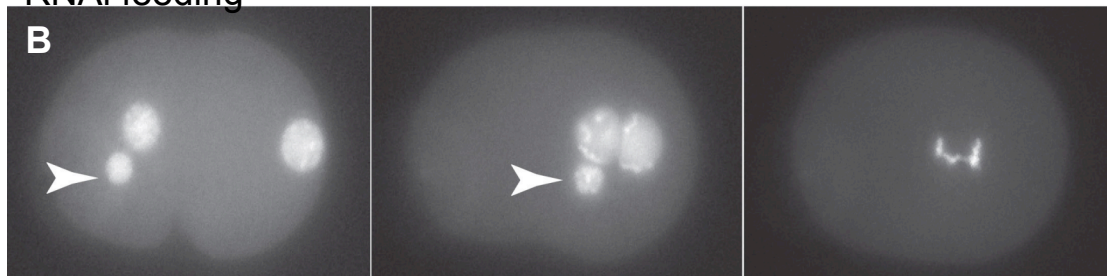


Fig.10

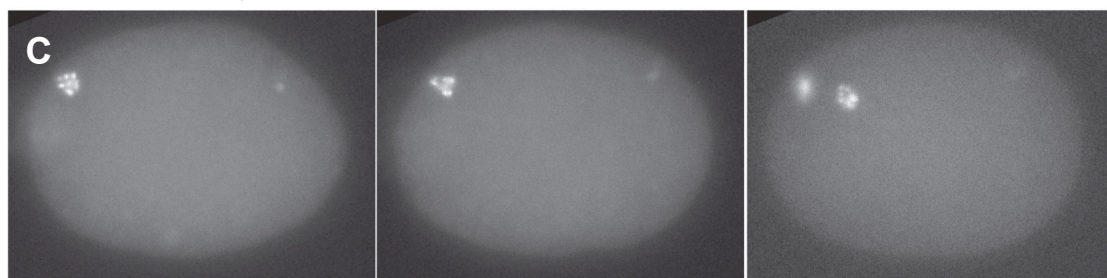
Wild Type



RNAi feeding



RNAi microinjection



**Fig. 10 Histone::GFP *cyp31A(RNAi)* embryos display severe defects during meiosis**

Images from time lapses (single frames were taken at ~10', 15', 30' after fertilization).

Wild type embryos (**A**). One polar body is visible at the anterior pole indicated by an **arrowhead** (the second is in a different focal plane).

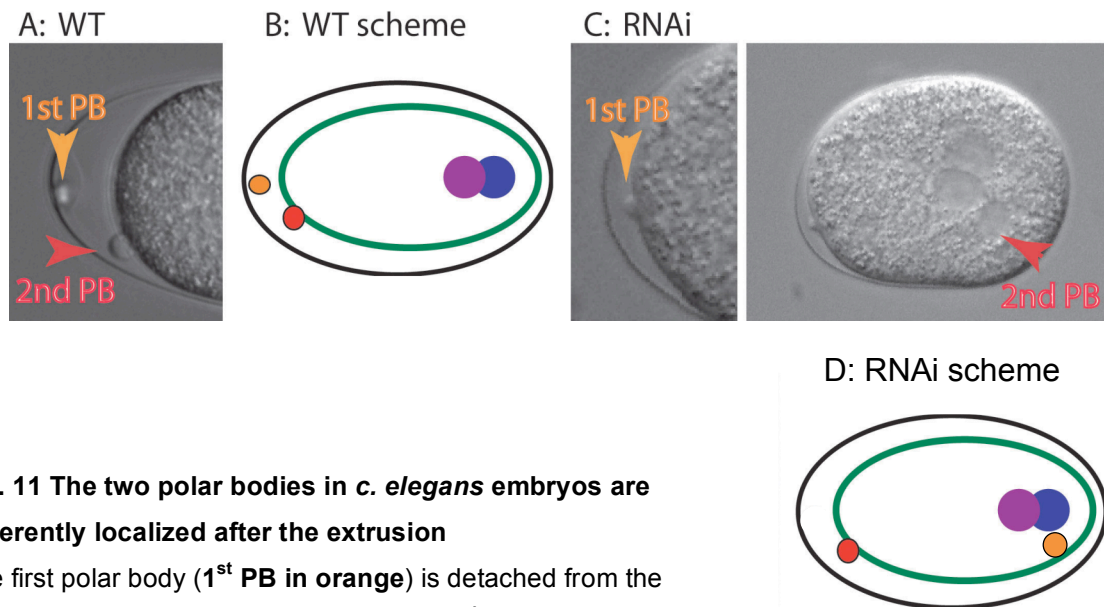
*cyp31A(RNAi)* embryos in which the silencing was performed by feeding (**B**): the reinserted polar body is indicated by **arrowheads**.

*Cyp31A(RNAi)* microinjected embryos: the development arrested before the accomplishing of meiosis (**C**).

In a wild type embryo, the first polar body contacts the eggshell and eventually is completely detached from the cell. The second polar body, instead, is localized on the plasma membrane of the just fertilized embryo (fig. 11 A, B). When RNAi against *cyp31A* was performed by feeding, embryos

presented only one polar body on the membrane (fig. 11 C) whereas the polar body that normally contacts the eggshell was not observed.

Fig. 11



**Fig. 11 The two polar bodies in *c. elegans* embryos are differently localized after the extrusion**

The first polar body (1<sup>st</sup> PB in orange) is detached from the embryo whereas the second polar body (2<sup>nd</sup> PB in red) stays in contact with the plasma membrane of the embryo.

An image with the wild type situation is presented in **A**.

The same images, as a scheme, is depicted in **B**.

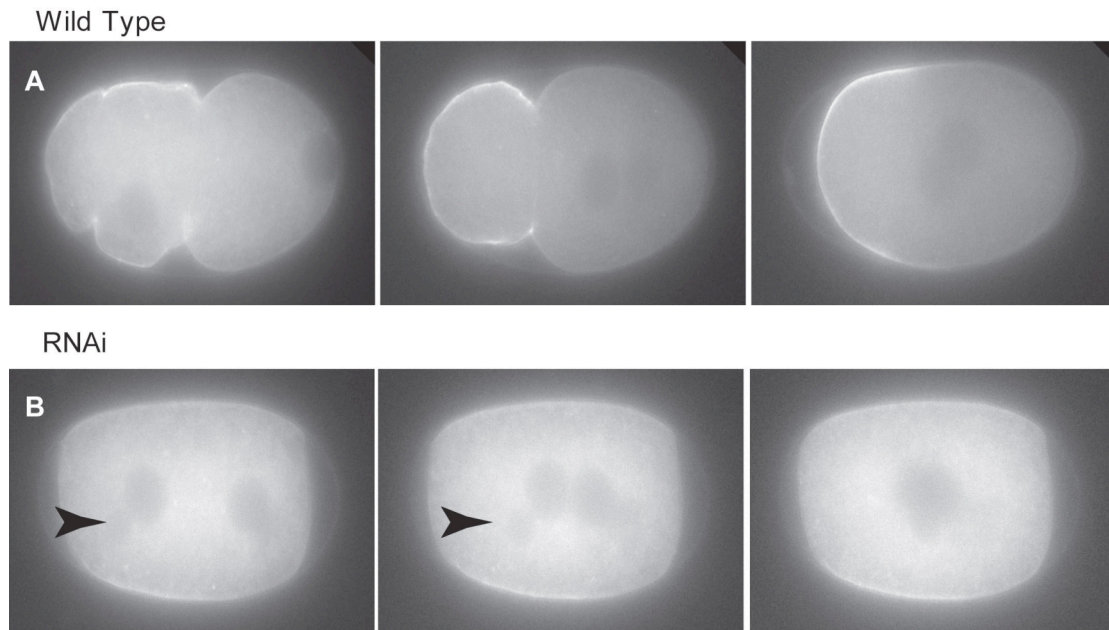
*cyp31A(RNAi)* embryos extrude the first polar body but this is not in contact with the eggshell (**C**). The second polar body enters in the cell and fuses with the *nucleus* (**C**).

The schematic representation is presented in **D**.

### 3.6 *cyp31A(RNAi)* embryos exhibit symmetric first division

Another defect observed in the time lapses with *cyp31A(RNAi)* embryos was an unnatural symmetric first division. In order to test the appropriate sorting of the PAR proteins we took additional time lapses using a PAR6::GFP fusion protein. PAR6::GFP line was used to test the polarization process at the anterior pole of the embryo (fig. 12 A).

Fig. 12



**Fig. 12 *cyp31A(RNAi)* embryos fail to segregate PAR6::GFP at the anterior pole**

Time lapses (single frames were taken at around 10', 15', 30' after fertilization).

In wild type embryos PAR6::GFP is restricted to the anterior pole (**A**).

In *cyp31A(RNAi)* embryos, PAR6::GFP localizes in the cytoplasm or at the cortex in a symmetric manner. Note the complete lack of pseudocleavage (**B**).

The polar body that is re-inserted in the cell is indicated by the **arrowhead**.

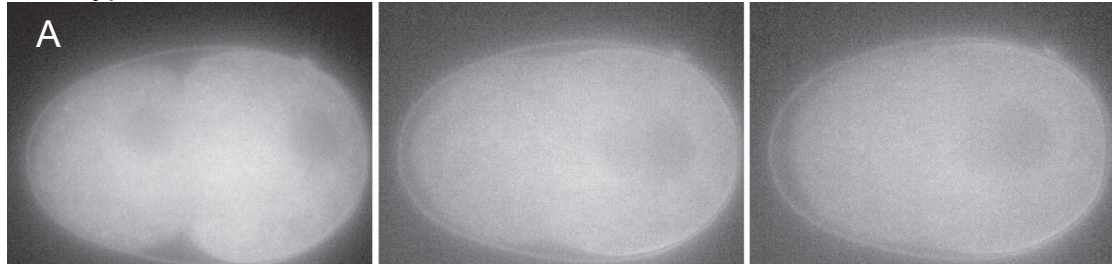
These time lapses illustrated that PAR6::GFP was able to move from the cytoplasm to the cortex (although not completely), but failed to accumulate at the anterior pole (fig. 12 B). In addition, the distribution of the protein was symmetric and it maintained a symmetrically around the embryo cortex.



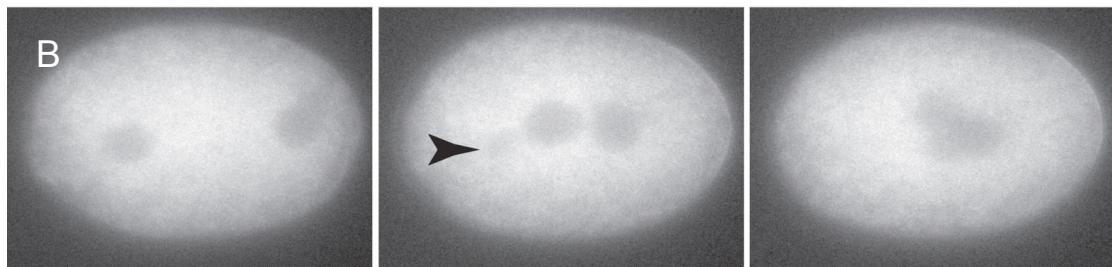
In order to examine if the polarization process of the embryo at the posterior pole was correctly accomplished, we recorded time lapses using a PAR2::GFP transgenic line.

Fig. 13

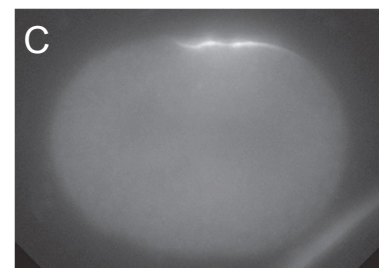
Wild type



*Cyp31A2(RNAi)*



*Cyp31A2(RNAi)*



**Fig. 13 *cyp31A(RNAi)* embryos fail to segregate PAR2::GFP at the posterior pole**

Time lapses (single frames were taken at around 10', 15', 30' after fertilization).

In wild type embryos PAR2::GFP is restricted to the posterior pole (**A**).

In *cyp31A(RNAi)* embryos, PAR2::GFP is localized in the posterior side of the embryo but it fails to expand, resulting in a little patch of protein. Note the complete lack of pseudocleavage (**B**). Alternatively PAR2::GFP is localized in a lateral manner (**C**).

The polar body in the cell is indicated by **arrowhead in B**.

In our experiments PAR2::GFP was able to reach the cortex, but its localization was observed in a very little patch (smaller than in the wild type) at the posterior pole or, alternatively, at the lateral edge of the embryos (fig. 13).

### **3.7 *cyp31A(RNAi)* embryos are osmosensitive**

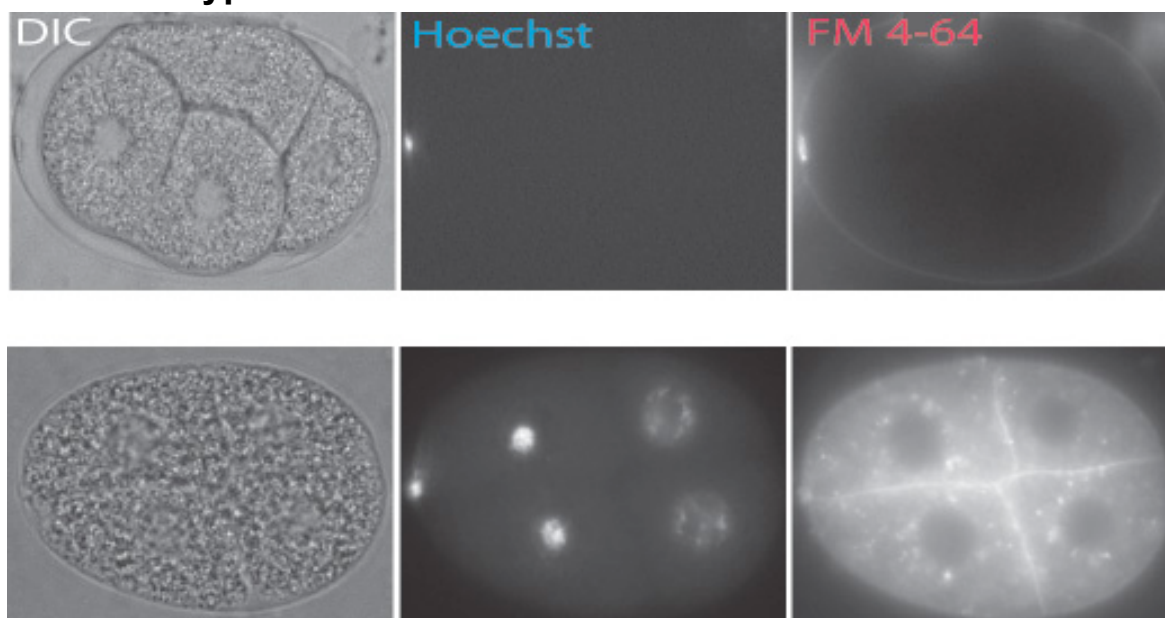
In addition to the defects described above, the *cyp31A(RNAi)* embryos in the time lapse analysis appeared enlarged. They occupied even the perimembrane space: the area that is between the eggshell and the embryonic plasma membrane. This observation suggested that these embryos might not be able to regulate their osmotic balance when the external salt concentration is altered and therefore, that *cyp31A(RNAi)* embryos are osmotically sensitive. This defect was so strong that, in some cases, during the process of taking movies, “bursting” of eggs in M9 buffer (the medium routinely used to handle *C. elegans*) was observed. The capability to modulate such osmotic equilibrium resides in the eggshell which, if appropriately constructed and functional, isolates the embryos from the external environment. The eggshell is a powerful barrier that prevents the embryo from being affected by changes in the surrounding milieu. However, if embryos with a defective eggshell are placed in hypertonic media (high salt concentration), the cells, once in contact with the medium, immediately respond by losing water and reducing their cell surface. This event leads to decrease in the cell volume and to a corresponding increase in the concentration of internal ions. The process stops when the internal and the external ion concentration are equal. The regulation of the cell volume is regulated by ion channels, transporters and pumps that modulate the intracellular concentration of water, ions and osmolytes in order to re-establish the cellular volume (for a review see Kwon and Handler, 1995). On the contrary, upon hypotonic stress, cells take up water and become enlarged. The regulation and the mechanism by which the cell regulates osmotic homeostasis still remains unclear. When embryos were depleted of CYP31A, they shrunk or swelled depending on the external salt concentration.

In order to test if the osmotic sensitivity was due to defects in the eggshell structure, a direct staining approach was used. Normally, dyes do not cross the barrier of the eggshell, but a “permeabilization procedure” is required. When the wild type embryos were imbedded in a solution with the dyes Hoechst 33342 (that stains DNA) and FM 4-64 (that marks the

membranes), only the DNA of the first polar body was stained. These dyes did not cross the innermost lipid (lipid-rich) layer that confers impermeability to the eggshell (fig. 14) (Foor W.E., 1967). In *cyp31A(RNAi)* embryos, on the contrary, the dyes penetrated the eggshell. FM 4-64 stained lipids of the membrane and also some punctuate structures (probably endosomes), whereas Hoechst 33342 stained the DNA of the polar bodies and of the nuclei. These results confirmed that the eggshell in *cyp31A(RNAi)* embryos is non functional and permeable to small molecules (fig. 14).

Fig. 14

**A: Wild type**



**Fig. 14** *cyp31A(RNAi)* embryos are permeable to dyes

A wild type embryo is stained with Hoechst 33342 and FM 4-64 (**A**).

In the same conditions, a *cyp31A(RNAi)* embryo, absorbs dyes and is stained (**B**).

In the wild type is possible to observe the emptiness in the peri-membrane space between the plasma membrane and the eggshell. The perimembrane space is filled up by the cell in *cyp31A(RNAi)* embryo.

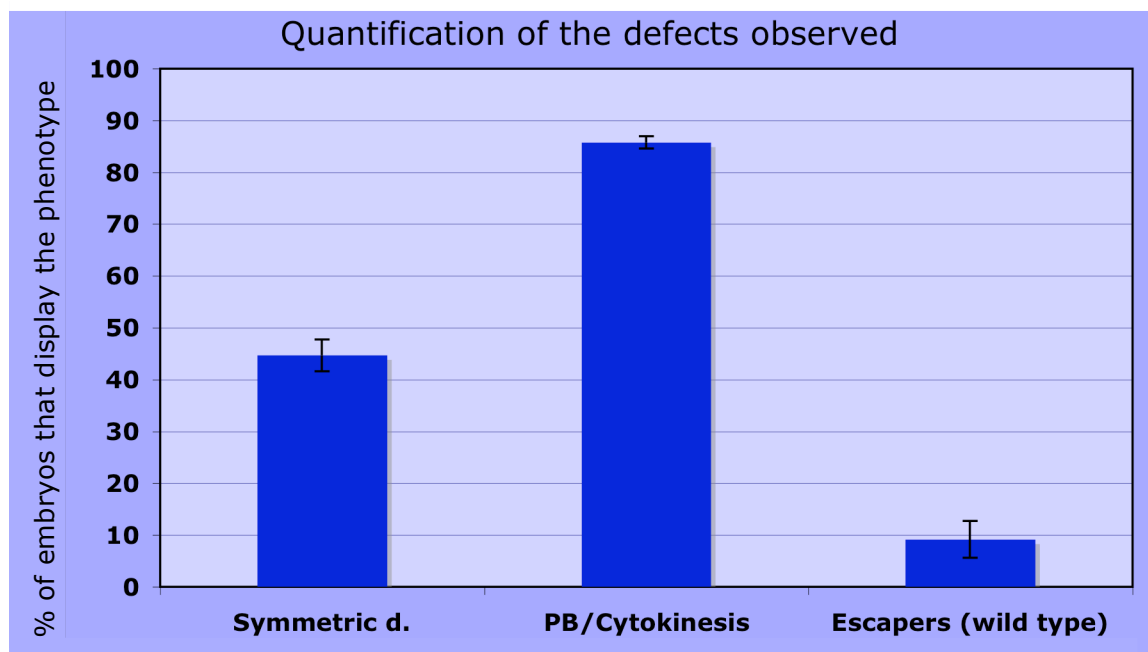
The permeability of the *cyp31A(RNAi)* was tested with other dyes (Nile red, Dil), too. These additional experiments confirmed the permeability of *cyp31A(RNAi)* embryos. The experiments were carried out in order to exclude the possibility that the observed permeability was caused by peculiar

chemical-physical properties of the dyes used in the experiment (data not shown).

### 3.8 Quantification of the defects displayed by *cyp31A(RNAi)* embryos

Additional *Cyp31A(RNAi)* embryos (between one and four cell stage) were observed in order to quantify the frequency of the three defects detected: all the *cyp31A(RNAi)* embryos were permeable to the dye Hoechst 33342 and enlarged in low salt concentration (osmotic sensitivity) (fig. 15). Failure in the extrusion of the polar body and defects in cytokinesis were observed in 85.75% of the embryos. A symmetric first mitotic division was detected in  $44.67 \pm 3,1\%$  of the embryos. RNAi escaper larvae with normal development were observed (9.16% of the eggs).

Fig.15



**Fig. 15 Quantification of the defects observed in *cyp31A(RNAi)* embryos**

The defects observed (symmetric cell division and polar body) are quantified in this graphic.

The analysis has been performed in embryos between one and four cell stage.

All the embryos were permeable to dyes.

The phenotypes described above led us to ascribe *cyp31A* to a class of genes named POD (Polarity and Osmotic Defects). At the moment, there are already six genes with similar phenotypes that are recognized by the Caenorhabditis Genetics Center (CGC). The complete name (using the three letter-one number code) of *cyp31A* gene would be *pod-7*. Therefore, in the following, I will refer to the gene *cyp31A* as ***pod-7***.

### **3.9 Analysis of genes that phenocopy *pod-7(RNAi)***

At first glance, the three traits detected in *pod-7(RNAi)* embryos (osmotic sensitivity, symmetric cell division and failure of extrusion of a polar body) seemed to be related to different cellular processes that exploit diverse protein machineries. However, a long list of single gene mutants and knock downs, was found in literature that display a similar phenotype to *pod-7(RNAi)* (table n.2).

The mutations and knock downs that led to the described phenotypes could be grouped by criteria of functionality in two major groups. The genes listed in the upper part of table n.2 (light green) code for subunits of the APC (Anaphase Promoting Complex), *separase* etc. For these proteins, there is a clear explanation for the meiotic defects observed, but the connection with osmosensitivity and the polarity establishment has not yet been unraveled.

A second group of proteins (highlighted in darker green in table n.2) contains genes such as *pod-1*, *pod-2* and *chs-1* that, in contrast with the first group, are not involved in meiosis.

-*Pod-2* encodes for the sole *C. elegans* homologue for acetyl CoA carboxylase: a key enzyme that catalyzes the first rate-limiting reaction in long chain fatty acid synthesis. When mutated, the embryos displayed failure in extrusion of the second polar body, osmosensitivity and symmetric first division (Rappleye C. A. *et al.*, 2003). The polarity defects (but not the osmotic sensitivity) could be rescued by addition of specific fatty acids: palmitic, stearic, oleic, linoleic, arachidonic acid to the media. (Rappleye C. A. *et al.*, 2003). For *pod-2* a role in the constitution of the lipid rich layer of the eggshell has been proposed.



Table n.2

<i>Genes</i>	<i>O.R.F.</i>	<i>Function</i>	<i>Polarity defects</i>	<i>Osmotic defects</i>	<i>Meiotic defects</i>	<i>Reference</i>
<b><i>Pod-3</i></b>	W10C6.1	APC subunit	X	X	X	100
<b><i>Pod-4</i></b>	F10C5.1	APC subunit	X	X	X	100
<b><i>Pod-5</i></b>	Y110A7A.17	APC subunit	X	X	X	100,113
<b><i>Pod-6</i></b>	F10B5.6	APC subunit	X	X	X	100
<b><i>Emb-30</i></b>	F54C8.3	APC subunit	X	X	X	35,100,129
<b><i>Ubq-2</i></b>	ZK1010.1	Ubiquitin	X	X	X	100
<b><i>Fzy-1</i></b>	ZK177.6	Cdc-20	X	X	X	100
<b><i>Sep-1</i></b>	Y47G6A.12	Separase	X	X	X	100
<b><i>Cyk-3</i></b>	ZK328.1	Ubiquitin hydrolase	X	X	X	35

<b><i>Chs-1</i></b>	T25G3.2	Chitin syntase	X	X	X	144
<b><i>Pod-1</i></b>	Y72A2B.1	Coronin-like	X	X	X	100,101,127
<b><i>Pod-2</i></b>	W09B6.1	AcetylCoA carboxylase	X	X	X	101,127
<b><i>unassigned</i></b>	F32H2.5	Fatty acid synthase	X	X	X	101
<b><i>Emb-8</i></b>	K10D2.6	NADPH-CYP	X	X	X	101,129
<b><i>Pod-7</i></b>	H02I12.8	Cytochrome P450	X	X	X	20, 35, 101 129

-*Pod-1* codes for a coronin-like protein that shows polarized localization at the anterior pole. Mutation of this gene causes a doubling of the eggshell lipid rich layer that leads to the osmotic sensitivity phenotype (Rappleye C. A. *et al.*, 1999).

-*Chs-1* (CHitin Synthase) catalyzes the polymerization of UDP-N-acetyl-glucosamine to produce chitin for the homonymous eggshell layer.

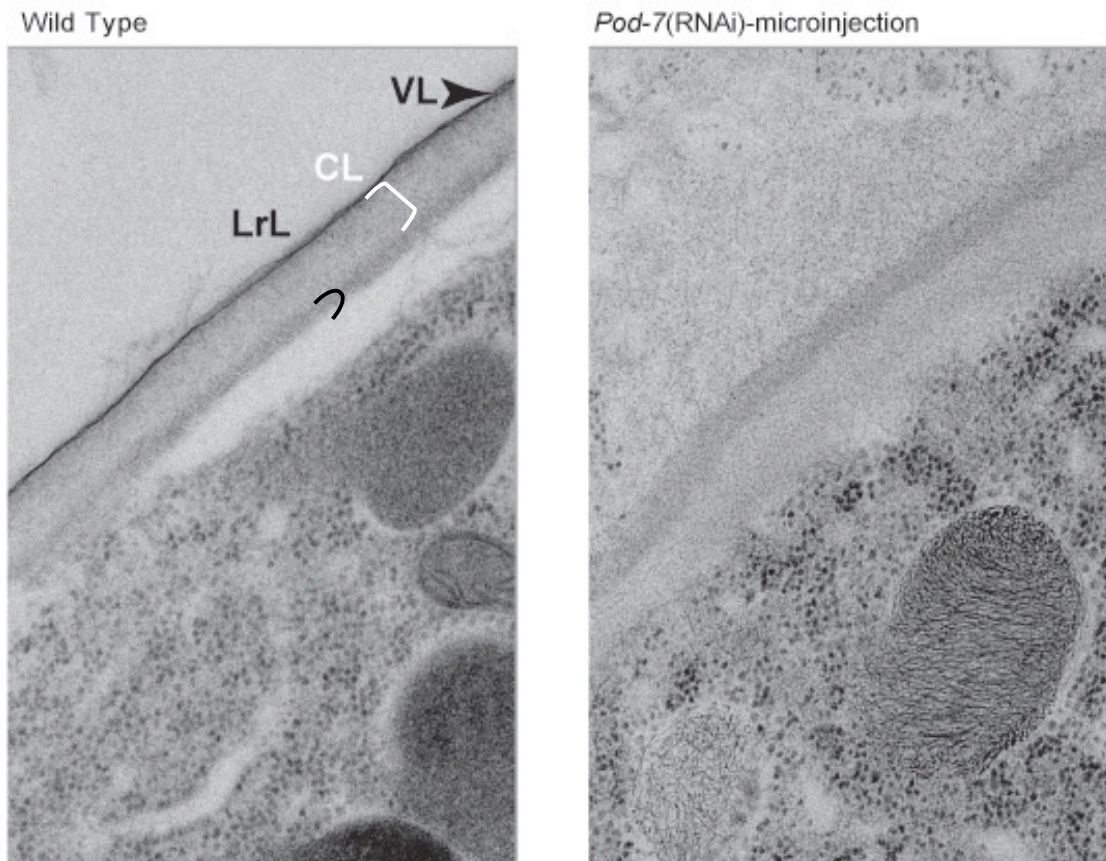
The fact that these genes, which potentially affect the synthesis of the eggshell, display phenotypes very similar to what we observed after *pod-7(RNAi)* led us to investigate the eggshell in more depth.

### **3.10 EM analysis detects a defective eggshell in *pod-7(RNAi)* embryos**

Analysis of the genes listed in the second POD subgroup suggested that the phenotypes of abnormal polarity establishment, failure in the extrusion of polar bodies and osmotic sensitivity are associated with a defective eggshell. In order to test whether POD-7 is involved (directly or indirectly) in the synthesis of the eggshell we observed by EM the three layers in *pod-7(RNAi)* embryos. In order to investigate the phenotype under conditions of the most severe depletion, EM analysis was carried out in eggs in which POD-7 dsRNA has been microinjected. *pod-7(RNAi)* embryos were imaged *in utero* because depletion of the cytochrome P450 resulted in eggs that were fragile and osmotically sensitive.

In the wild type eggshell, the most external vitelline layer (left in the figure) has the appearance of a continuous, dark and sharp line (VL), the underlying chitinous layer (CL) was seen as a clearer strip (light grey), and the innermost electron dense stratum was identified as the lipid-rich layer (LrL). These three sheets were followed by an empty space (white) that, with irregular thickness, surrounded the plasma membrane of the embryo. EM images of *pod-7(RNAi)* embryos showed that the three well organized layers present in the wild type eggshell were completely misarranged after depletion of CYP31A (fig. 16). In *pod-7(RNAi)* eggs the vitelline layer was completely absent and, in the rare cases in which it was present, it appeared as an uncontinuous and fuzzy structure. The sheets that were underneath the vitelline layer showed even more drastic changes: the most external layer was dark (when usually it is the lightest one) and it was thinner than the wild type chitin layer. The innermost layer lipid rich layer, (that typically is darker) was, instead, lighter and with variable thickness. Finally, the empty perimembrane

Fig.16



**Fig. 16 Electron microscopy analysis shows that *pod-7(RNAi)* embryos have a defective eggshell**

Images of wild type embryo (left) shows the typical well defined three layers structure: Vitelline layer (VL- **white arrowhead**), Chitine layer (CL, **white parenthesis**), Lipid-rich layer (LrL, **black parenthesis**).

Beneath the lipid rich layer it is possible to observe, (only in the wild type), a white empty perimembrane zone.

*pod-7(RNAi)* embryos (right) displays a disorganized arrangement of the layers constituting the eggshell.

space between the eggshell and the embryo was undetectable. An explanation for the lack of perimembrane space is that the osmotically sensitive embryos are enlarged and thus they fill up this empty space (see also fig. 14). The EM analysis confirmed that the aberrations detected in *pod-7(RNAi)* embryos could be ascribed to defects in the composition and/or appropriate construction of the eggshell and that *pod-7* is involved in the regulation and/or in the synthesis of this structure.

Unfortunately, due to the lack of information concerning the composition of the eggshell, it was not possible to stain in EM all three layers. This experiment could have allowed us to assess whether *pod-7(RNAi)* embryos are missing one specific layer, or if exists a more general problem in the assembly of the three layers.

### **3.11 Functional studies of a WT eggshell**

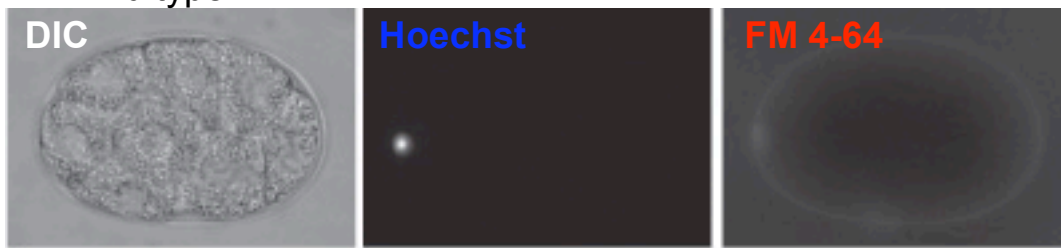
Published data concerning the function of the three layers of the eggshell is scarce and, in some cases, conflicting, thus, we decided to carry out a functional study of the wild type eggshell. No data has been reported concerning the putative function of the external vitelline layer. This layer can be easily removed by brief bleaching with hypochlorite without any effect on the further development of the embryo and without morphological changes in the structure of the egg (fig. 17). The chitin layer can be removed by treatment with chitinase. This layer is the one that confers shape to the egg. After removal of the chitine layer, the embryo becomes rounded and fragile and should be kept in isotonic medium (Wolf N. *et al.*, 1983). The medium that we used in order to avoid osmotic shock was the EGM (Embryonic Growth Medium) that was established as medium to culture single embryonic blastomeres (Edgar L.G., 1995).

When the two procedures with hypochlorite and chitinase were performed on wild type eggs, only the lipid-rich layer was left to surround the embryos. The absence of the chitin layer was indicated by rounding up of the embryo. The lipid-rich layer is the stratum that determines the impermeability of the eggs. When we performed staining of these embryos, the dyes were unable to penetrate this layer (fig. 17).

The evidence that wild type embryos after bleaching and chitinase treatment were impermeable to dyes and the observation that the same dyes were able to cross the eggshell of untreated *pod-7(RNAi)* embryos (fig. 14);

Fig. 17

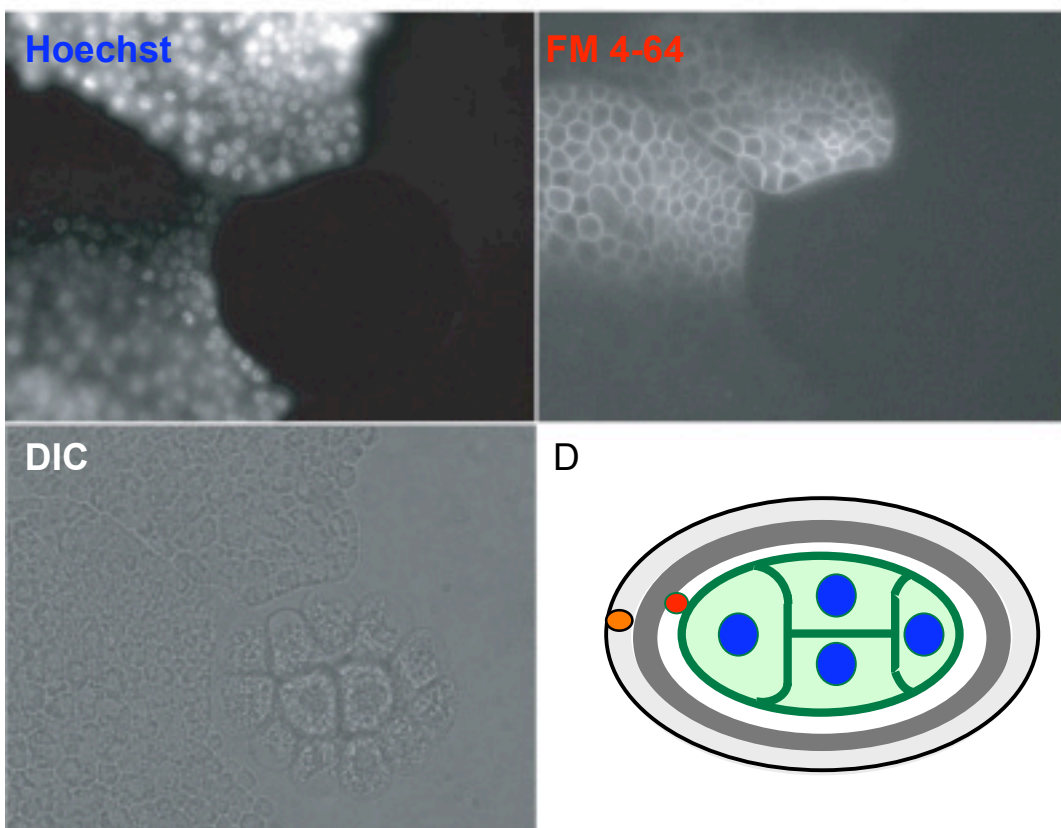
A- Wild type



B- Wild type after bleaching (removal of the VL)



C- Wild type after bleaching and chitinase treatment



**Fig. 17 Functional study of the three layers of a wild type eggshell**

Wild type embryo stained with Hoechst 33342 and FM4 64 (A).

Wild type embryos after bleaching (note the disappearance of the first PB) (B).

After chitinase treatment the embryos rounds up but are still impermeable (C).

Schematic representation of the eggshell structure: black line VL, light gray CL, dark gray LrL, Orange 1<sup>st</sup> PB, Red 2<sup>nd</sup> PB, green plasma membrane (D).

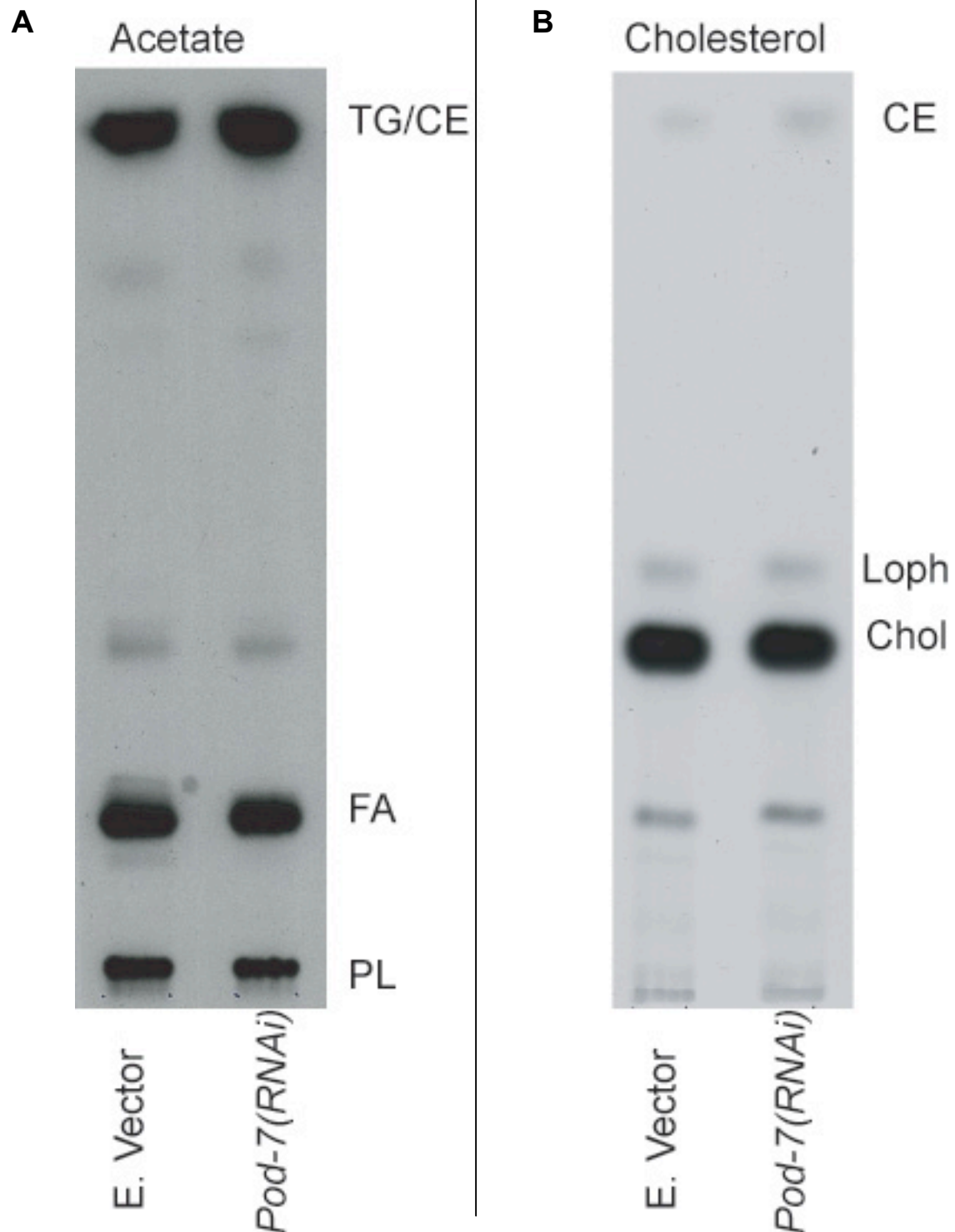
demonstrates that embryos depleted of POD-7 are unable to synthesize a functional lipid rich layer. The embryos treated with hypochlorite and chitinase, on the other hand, were fragile and very sensitive to sudden changes in salt concentration in the media. When these wild type eggs were exposed to a lower salt concentration (KCl 50 mM), they responded to the hypotonic media by increasing cell size and eventually exploding (data not shown). Moreover, untreated *pod-7(RNAi)* embryos exploded when exposed to hypotonic conditions. This additional information indicated that the presence of the lipid-rich layer is not enough to ensure correct regulation of osmotic balance. Altogether this data demonstrated that, in addition to defects that can be ascribed to the lipid-rich layer, other problems reside in the layer that regulates osmotic balance and in general assembly of the three layers (fig. 16).

Additional information coming from these experiments was that the first polar body, (the one that is detached from the embryo) (fig. 11), is inserted in the eggshell externally to the impermeable lipid-rich layer (because it absorbed the dye hoechst). This polar body is located at the level of the vitelline layer (because was lost after hypochlorite treatment) (fig. 17).

### **3.12 Characterization of the metabolic product of POD-7.**

As a first attempt to identify the metabolic product of POD-7 we performed a series of experiments in which molecules that can be metabolized by POD-7, radioactively labeled with  $C^{14}$  or  $H^3$ , were mixed with the bacteria fed to the worms. *C. elegans* took up and metabolized these compounds since the labeling was transferred to the lipids produced by the modification of the labeled molecules (fig. 18). Our expectation for this experiment was that, after silencing of an enzyme such as a CYP, the substrate that can not be modified should accumulate and the metabolic product should disappear. The lipids were radioactively labeled with acetate (fig. 18 A) or cholesterol (fig. 18 B), extracted from adult gravid worms and separated by TLC (Thin Layer Chromatography).

Fig. 18



**Fig. 18 Comparative analysis of empty vector and *pod-7(RNAi)*'s lipid extract do not show differences**  
Autoradiographic films of the TLC on which were resolved lipid extract from worms fed either with empty vector bacteria (E. Vector) (1<sup>st</sup> line) or *pod-7(RNAi)* (2<sup>nd</sup> line).  
The bacteria was mixed with radioactively labeled acetate (A) or cholesterol (B).  
PhosphoLipids (PL), Fatty Acid (FA), TriGlycerides (TG), Cholesterol Esther (CE), Lophenol (Loph).



Comparison of the autoradiographic pattern of lipids extracted from gravid worms, fed with either the empty vector or with a plasmid producing the dsRNA, did not reveal any evident or reproducible variations that could elucidate the substrate of POD-7. The experiments were repeated under different conditions and TLCs were resolved by different mobile phases without observing any relevant dissimilarities. This result could be explained by the assumption that the metabolic product of POD-7 is required at low amounts, therefore, the differences in metabolism are below the resolution of an autoradiography. An alternative explanation could be that the substrate and the product of POD-7 might have similar chemical and physical properties. In this case, the unmetabolized substrate would migrate together with the product without any variation in the spots detected on the film. For instance, fatty acids that differs in the position of a double bound migrate with the same pattern, thus depletion of an enzyme that modifies the position of the double bonds would not give a significant shift when compared with the pattern found in the wild type worm extracts.

### **3.13 The embryonic lethality of *pod-7(RNAi)* embryos can be rescued by dietary supplementation of lipidic extract**

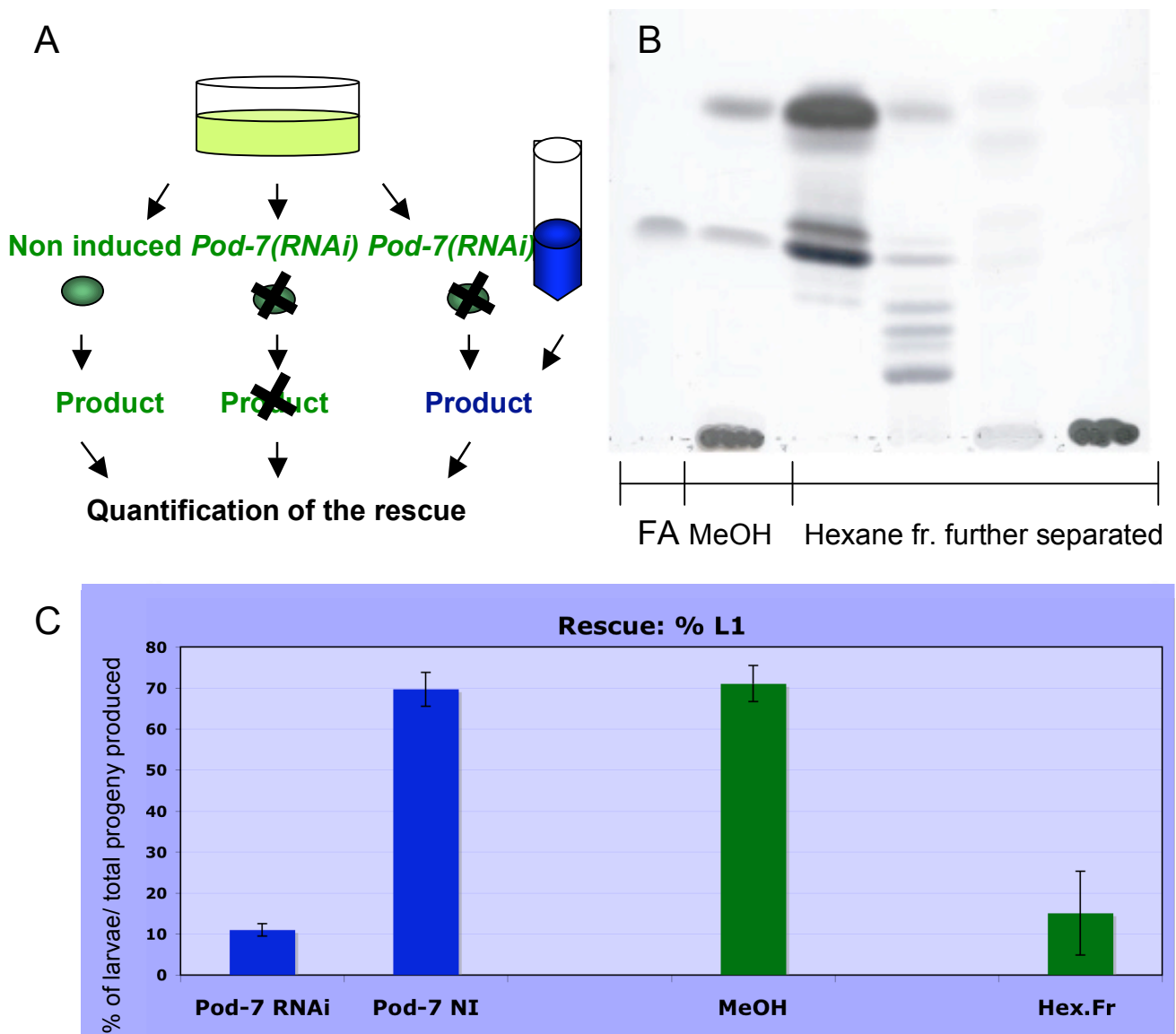
In several studies, the defects observed in mutants or RNAi worms have been rescued by the addition of specific lipids into the media (Watts J.L. and Browse J., 2006; Rappleye C. A. *et al.*, 2003; Motola D.L. *et al.*, 2006). We decided to use this approach to identify extracts that contained the lipid product of POD-7. We set up a bioassay in order to test and purify fractions of lipids that rescue the embryonic lethality caused by *pod-7(RNAi)*. *pod-2(ye60)* embryos, displaying phenotypes similar to *pod-7(RNAi)* were (partially) rescued by supplementation of specific FA. As a first attempt we tested the activity of these specific fatty acids: oleic, elaidic,  $\gamma$ -linoleic, palmitic and stearic acid in *pod-7(RNAi)* embryos (Rappleye C. A. *et al.*, 2003). Unfortunately, no effect of these fatty acids was observed in our bioassay (data not shown). This lack of rescue were not due to the toxic effects of fatty



acids as such, whose nematocide activity has been reported (Stadler M. *et al.*, 1994), as several concentrations were tested without any change in activity.

Given that fatty acids failed to rescue *pod-7(RNAi)* phenotype, we decided to test with the bioassay lipids directly extracted from the worms. The characterization of the embryonic lethality and the absence of effects in the mother showed that the metabolic product of POD-7 could be specifically required during the embryonic development. In order to enrich the extracts with the metabolic product of POD-7 we cultured *C. elegans*, bleached the gravid mothers in hypochlorite, and extracted the lipids from the embryos obtained. The lipids were extracted from these embryos with methanol. This original methanol fraction was re-extracted with an equal volume of hexane (the two solvents formed two different phases). As a general rule, the most hydrophobic lipids (for instance, sterols or triglycerides) partitioned in the upper hexane fraction whereas the most hydrophilic moved into methanol (fig. 19). When added to *pod-7(RNAi)* worm cultures, only the methanol fraction was able to rescue the developmental arrest of the embryos suggesting that it contained the metabolic product(s) of POD-7. However, we could not rule out the possibility that, if POD-7 is in the middle of a pathway that includes several lipid-modifying enzymes. In this case, all the molecule(s) produced downstream POD-7, and not exclusively the metabolic product of this CYP, could rescue *pod-7(RNAi)* defects.

Fig. 19



**Fig. 19 Methanol fraction can rescue the embryonic lethality induced by *pod-7(RNAi)*.**

Scheme of the bioassay (**A**).

Lipids extracted by *C.elegans* worms were separated into methanol (MeOH) and hexane fractions (the later has been further separated into 4 additional subfractions) and eventually loaded in a TLC (**B**).

Result of the bioassay with quantification done by counting the number of larvae/ total progeny produced (**C**).

### 3.14 Analysis of other proteins potentially involved in the metabolic pathway that requires POD-7

With the intention of investigating whether other genes that display POD phenotypes could be involved in the pathway that includes POD-7, we analyzed possible candidates (table 2):

-The genes *pod-2* (ORF W09B6.1) and *FAS* (ORF F32H2.5) code for the first two enzymes responsible for the biosynthesis of fatty acids. However, when RNAi was performed to silence these genes, the defects observed were not only at the level of the progeny. Fatty acids are required during all the developmental stages and the lack of these enzymes leads to several defects during the first generation (in addition to embryonic lethality in the second generation). The exhibition of all these defects, in contrast with the temporally specific defects observed in *pod-7(RNAi)*, suggests that *pod-2* and *FAS* are upstream of POD-7 in the pathway.

In addition to POD-2, another protein, functionally related with CYPs, was reported to have a similar phenotype when mutated: the NADPH cytochrome P450 reductase (NCPR) EMB-8. A NCPRs is the protein that supplies electrons to the CYPs for their oxidative reactions (Backes W.L. and Kelley R.W., 2003). *emb-8* (ORF K10D2.6) is a member of this class of proteins and is conserved through evolution (64% similarity with the human NCPR). For this gene, several mutant alleles have been characterized:

*emb-8(hc69)* is a thermo-sensitive, mutant. The transfer of *emb-8(hc69)* gravid worms at 25° C degree causes embryonic lethality of the progeny. This embryonic lethality is caused by osmosensitivity and symmetric division (in about half the cases) (Rappleye C. A. *et al.*, 2003). The arrested embryos produced were morphologically indistinguishable from *pod-7(RNAi)*. *emb-8(hc69)* has been shown to affect, for instance, PIE-1, (Tenenhaus C. *et al.*, 1998) GPL-1, (Crittenden S.L. *et al.*, 1997) as well as PAR proteins distribution (Rappleye C. A. *et al.*, 2003). Despite the availability of a mutant to study this protein, the cytochrome P450 that exploits the electrons supplied by EMB-8 has not yet been identified.

The hypothesis that we wished to test is whether POD-7 might be the CYP that accepts electrons supplied by EMB-8 i.e. if these two proteins co-operate to carry out the same reaction in the pathway. The description of the

phenotypes of *emb-8(hc69)* mutants indeed coincided perfectly with what was observed in *pod-7(RNAi)* embryos (see table 3). Furthermore, when searches through databases of RNAi screens were performed in order to find additional knock-downs/mutants that produce similar phenotypes, a total of 59 ORFs were found (<http://wormbase.org/db/searches/basic?class=Any;query=osmotic%20;Search=Search;offset=0&scroll=+50>). Nevertheless, among them, no cytochrome P450s, with the exception of *pod-7*, were present in the list.

This data strengthened the argument that the NCPR EMB-8 could assist POD-7 with its metabolic reactions.

The similarities between the mutant *emb-8(hc69)* and *pod-7(RNAi)* resided not only in the general features displayed, but also in its capability to localize specific polarity markers in a similar manner. When the localization of specific markers for polarity was investigated (PAR-3 for the anterior and PAR-2 for the posterior) in *emb-8(hc69)*, PAR-3 was detected around the cortex whereas PAR-2 was found in a little patch, in a lateral fashion or at the

Table n.3

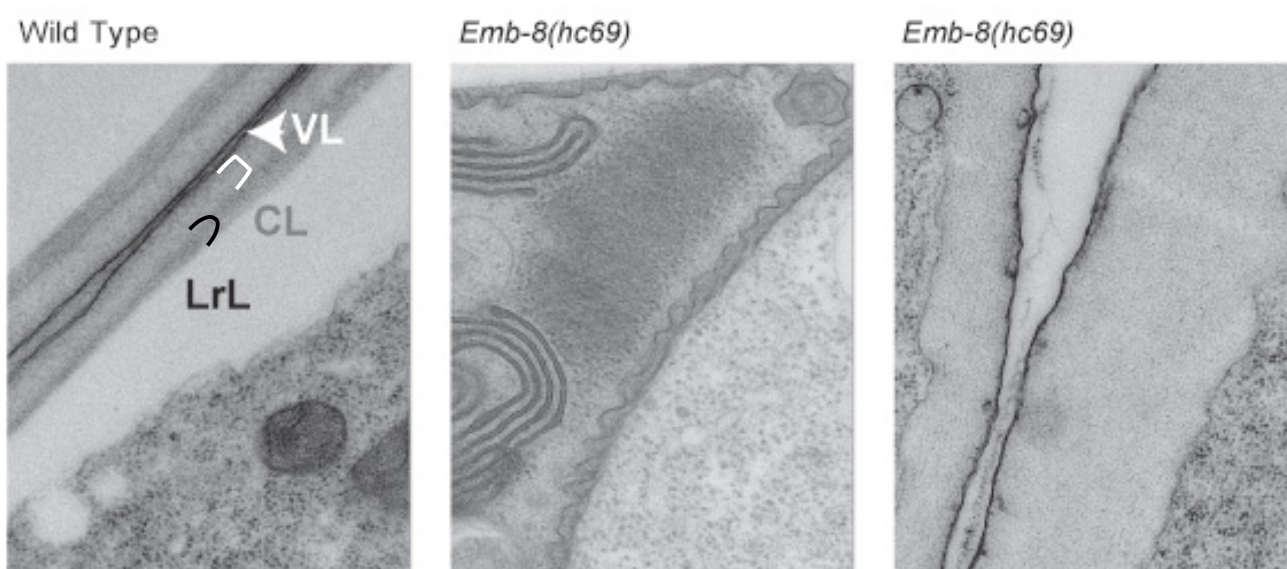
<b>Phenotype observed</b>	<b><i>Emb-8(hc69)</i> (Ref. )</b>	<b><i>Pod-7(RNAi)</i></b>
Osmotic sensitivity	100%	100%
Symmetric division	50%	44.7±3.1%
Anterior polarity marker	PAR-3 (immunostaining) all around the cortex	PAR-6::GFP all around the cortex
Posterior polarity marker	PAR-2 (immunostaining) Posterior little patch/lateral	PAR-2::GFP Posterior little patch/lateral
Extrusion of both PB	Not observed	Not observed

posterior pole (Rappleye C. A. *et al.*, 2003). This situation coincided entirely with the data that we reported in this study for *pod-7(RNAi)* embryos (fig. 12 and 13).

With the intention of verifying whether *emb-8(hc69)* also displays a defective eggshell we investigated its morphology by EM (fig. 20).

The result of the EM showed that the *emb-8(hc69)* mutant has a defective eggshell (fig. 16). The defects observed in the eggshell of these mutants are more severe and with a higher range of variability than the one displayed by *pod-7(RNAi)*. The three misarranged layers often varied with different patterns, even within the eggshell of the same embryo. The different patterns observed in the EM analysis might be explained by the diverse methods used for the protein depletion. *emb-8(hc69)* is a thermosensitive mutant and probably the shift to non permissive temperature led to more

Fig. 20



**Fig. 20 Electron microscopy analysis shows that of *emb-8(hc69)<sup>TS</sup>* embryos have a defective eggshell**

Images of wild type embryos show the typical well defined three layers structure: Vitelline layer (**VL- white arrowhead**), Chitine layer (**CL, white parenthesis**), Lipid-rich layer (**LrL, black parenthesis**) (**A**).

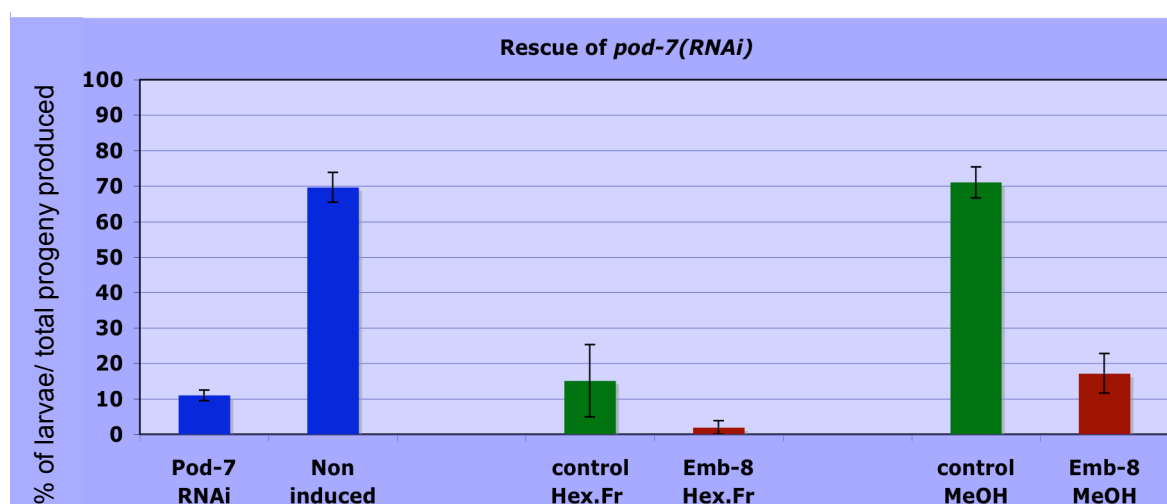
*emb-8(hc69)* embryos display a plethora of different configurations of misarranged eggshells: in some cases only an external undefined ruffled layer was present (**B**).

In other images *emb-8(hc69)* embryos present two external layers, but the lipid-rich layer is not observed (**C**).

penetrant silencing than the one obtained by the silencing of *pod-7(RNAi)*, the stronger depletion may cause a more dramatic morphological phenotype.

In order to test with a biochemical approach if these two proteins are involved in the same pathway, we decided to combine the bioassay that was previously established with lipid extract from the *emb-8(hc69)* mutant. In the bioassay we tested the extract from embryos derived from *daf-22(m130)* control worm population, (the lipid content of this mutant is comparable to the wild type). Simultaneously, *emb-8(hc69)* were cultured at non-permissive temperature and the embryos obtained were extracted following the same protocol.

Fig.21



**Fig. 21 Extracts from control worms, but not from *emb-8(hc69)* mutant, rescue the embryonic lethality caused by *pod-7(RNAi)***

Quantification of the rescue in the bioassay: the percentage of *larvae*/total progeny is reported in the graphic.

Embryonic lethality (*pod-7(RNAi)*), Non induced production of dsRNA (**Non Induced**).

*pod-7(RNAi)* with extract from control worms (**green**), from *emb-8(hc69)* (**red**).

The fact that the methanol fraction from *daf-22(m130)* (control worms) rescued *pod-7(RNAi)* whereas the effects of the *emb-8(hc69)* fraction were comparable to the inactive hexane fractions strongly suggested that EMB-8 might act in the pathway that requires POD-7. Since the *emb-8(hc69)* embryos seemed not to contain the POD-7 metabolic product (Fig. 21). Nevertheless, this assay can not exclude the possibility that, in addition to

POD-7, EMB-8 could have alternative partners in different metabolic pathways.

In conclusion, the phenotypes observed place the NCPR EMB-8 enzyme in a pathway that regulates polarity and osmotic defects along with POD-7. The biochemical data from the bioassay suggested that EMB-8 mutants do not contain the metabolic product the POD-7 (because they are not able to rescue *pod-7(RNAi)* phenotype) thus EMB-8 might act upstream (or more likely at the same level) of POD-7.

### **3.15 Future prospectives**

The partial purification of the methanol fraction gives us the possibility to further characterize the embryonic extracts (focusing on fatty acids) by HPLC-MS. In order to characterize the reaction and the metabolic product of EMB-8 (and POD-7), it will be particularly interesting to analytically compare the chromatographic profiles of extracts obtained by *emb-8(hc69)* and *daf-22(m130)*, to observe which classes of molecules accumulate (possible substrates) and which disappear (metabolic products). The characterization, quantification and analysis of the lipids extracted from *daf-22(m130)* is, at the moment, in progress.

## 4- DISCUSSION

### 4.1 A defective eggshell causes osmotic sensitivity in *pod-7(RNAi)* and *emb-8(hc69)* *C. elegans* embryos

Lipids, together with proteins, regulate essential metabolic processes. In order to achieve an overview of the cell metabolism, a clear understanding of lipid-based processes is required. Physiological function of lipid-modifying enzymes is a significant issue. In our project we have described and characterized the effects of the depletion of one of such enzymes: the *C. elegans* cytochrome P450, POD-7. When POD-7 is silenced, *C. elegans* embryos display several defects, starting with the first mitotic division and, eventually, leading to developmental arrest. It has been estimated that between 100 and 400 maternal proteins are needed for the first mitotic division (Gönczy P. *et al.*, 1999), whereas it is impossible to estimate the number of lipids required in this process. Not all of the proteins essential for the first cell division have been identified or characterized, and important phenomena such as cell polarization and the consequent fate decisions, still need to be investigated more extensively. The identification of novel proteins and lipids involved in the processes that take place at the beginning of development is important in order to fill in this gap of information.

NADPH Cytochrome P450 reductases are proteins that supply to CYPs such as POD-7, the electrons required for their metabolic reaction. Several previous studies have described that the localization of specific polarization markers: PIE-1 (Tenenhaus C. *et al.*, 1998), GPL-1 in the P granules (Crittenden S.L. *et al.*, 1997) and PAR proteins (Rappleye C. A. *et al.*, 2003) requires the NADPH Cytochrome P450 reductase EMB-8. However, the reason why EMB-8 is necessary for the establishment of polarity has not been clearly understood. In this investigation we demonstrate that EMB-8 contributes to the metabolic pathway in which cytochrome P450 POD-7 functions. POD-7 (co-operating with EMB-8) synthesizes lipid(s) that is essentially required for a functional eggshell.

The analysis of POD mutants suggests that a functional eggshell is indispensable for the correct establishment of polarity and to the regulation of



the osmotic balance. We report here for the first time EM images that show that the mutant *emb-8(hc69)* and *pod-7(RNAi)* embryos, have a defective eggshell. Furthermore, we have characterized the functions of the three layers that compose a wild type eggshell. Altogether, these data show that the osmosensitivity in *emb-8(hc69)* and *pod-7(RNAi)* embryos is caused by the incorrect assembling of the three layers of the eggshell.

#### **4.2 Several osmotic sensitive mutants display defective establishment of polarity**

The experiments described in this thesis permit to state that a defective eggshell is responsible for the osmotic sensitivity observed in *pod-7(RNAi)* embryos. The relationship between osmotic sensitivity and establishment of polarity has not been completely clarified. The polarization of a monocellular embryo is achieved through re-localization of specific proteins and is necessary to give rise to a normal and asymmetric first cell division.

Several mutants display Polarity and Osmotic Defects (which is why they are called POD). These observations suggest that the two phenomena might be linked. However, several other mutants exhibit osmotic sensitivity, coupled to a normal and asymmetric first division, result of the correct polarization of the embryo. One of the mutants in which this characteristic is encountered is an alternative allele of the already described *emb-8(hc69)<sup>TS</sup>* mutant. This second mutant, (*emb-8(t1462)*), divides asymmetrically in spite of being the osmotically sensitive (Rappleye C. A. *et al.*, 2003). The observation that two different mutations on the same gene can separate polarity from osmotic defects demonstrates that the two *phenomena* may not be connected by a simple relationship of cause and effect. Additional mutants have been reported to display osmotic but not polarity defects: *Emb-14*, *Emb-20* (Isneghi E. *et al.* 1983), *Csg-1*, *Csg-3*, *Csg-6*, *Gene17*, *Cta-3*, *Gene 04*, *Gene 21* (Gönczy P. *et al.*, 1999). The characterization of these mutants would be important in understanding the link between the regulation of polarity and osmotic balance. Unfortunately, none of these mutated genes have been identified and mapped, thus proteins coded by them are still unknown. Without this genetic information, it is not possible to study the function of the

proteins or to understand how these two mechanisms are associated with each other. Moreover, similarly to *emb-8*, *csg-1* presents multiple mutants alleles, but only one displays osmotic sensitivity and polarity defect whereas the other two show only osmotic sensitivity (P. Gönczy personal communication). The differences in the phenotypes observed in *emb-8* and *csg-1* can be ascribed to the different impact that the mutation might have on the proteins function. The possibility that the degree of activity of the protein can produce osmosensitivity and polarity defects or only the former, suggests that a complex system regulates the two processes.

An additional example of this complexity is *emb-20(g27)*. This mutant, as well as displaying osmotic sensitivity shows polarization defects. However, in contrast to *pod-7(RNAi)*, *emb-20(g27)* presents the proper localization of the polarity markers in the one cell-stage, whereas at a later stage of development (at around 28 cells) polarity defects can be detected (Tenenhaus C. *et al.*, 1998). The mutation of *emb-20(g27)* has not yet been mapped and, therefore rendering difficult to hypothesize the function of its protein and why the defects in the two processes can arise at different stages of development. Until all the proteins that control osmotic balance and establishment of polarity are identified and the mechanisms of the respective processes are understood, it will be difficult to clarify the relationship between osmosis and polarity. In this context, the characterizations of EMB-8 and POD-7 and their lipid products will be important to elucidate the two processes.

#### **4.3 The two processes of polarity establishment and adjustments of cell volume, both require the proper modulation of the cytoskeleton**

In this project we have demonstrated that *emb-8(hc69)* and *pod-7(RNAi)* embryos have a defective eggshell. In the wild type, the eggshell protects the worm from changes of the salt concentration in the surrounding media, whereas in osmotic sensitive embryos, the defective eggshell forces the cell to adjust its size to the external salt concentration (fig. 9 to 14 ).

In general, the regulation of cell volume under osmotic stress and establishment of polarity require the correct modulation of the same protein machinery, that is the cytoskeleton (Munro E. *et al.*, 2004; Rappleye C. A. *et*

*al.*, 1999). Because the cytoskeleton might be the connection between the defects observed in *emb-8(hc69)* and *pod-7(RNAi)*'s embryos, I will describe the remodeling process of the cytoskeleton during the early stages of development. Furthermore, I will compare the wild type and *pod-7(RNAi)* development in order to understand the effects of the silencing of POD-7 and EMB-8. Just after fertilization, according to one of the most accepted developmental models, the embryo cortex is under tension generated by a meshwork of actomyosin contractile units in a symmetric pattern. The polarization process of the embryo is initiated by the entering of the sperm that releases the tension (for a review see Cowan C.R. and Hyman A.A., 2004). Oocytes fertilized by anucleate sperm undergo normal meiotic division and eggshell synthesis (Sadler P.L. and Shakes D.C., 2000). This observation suggests that not the sperm pronucleus, but rather its centrosome is responsible for the initiation of the polarity establishment (Cowan C.R. and Hyman A.A., 2004b). As a consequence, the point at which the sperm enters, releasing the initial tension, acquires markers of posterior polarity (also if oocytes are "unnaturally" fertilized through the anterior pole) (Goldstein B. and Hird S.N., 1996). By analyzing in detail the phenotypes displayed by *pod-7(RNAi)* embryo, we can rule out the possibility that the defects are caused by the failure in the initiation of the polarization process. This is because the paternally contributed centrosome is identical to the wild type since RNAi does not affect the sperm cell (S. Ward personal communication).

After the initiation of the polarity establishment in the embryo, proteins that are polarity markers are relocalized to the poles through the movements of the actin cytoskeleton. This event confers to the two poles the characteristics of anterior or posterior. The regulation of these movements is based upon phosphorylation of the light chain of myosin by the Rho-associated Kinase (ROCK) (Amano M. *et al.*, 1996; Kureishi Y. *et al.*, 1997) or myosin light chain kinase (Frearson N. and Perry S.V., 1975). *C. elegans* embryos depleted of either Non-muscle Myosin (NMY-2) or its regulatory light chain (MLC-4) fail to establish a polarity. *Nmy-2* and *mhc-4* mutant embryos show a small patch of cortical PAR-2 at the posterior pole. This localization of PAR-2 proteins is accomplished by an actin independent mechanism but afterwards the posterior domain fails to expand and the embryos eventually

divide symmetrically (Shelton C.A. *et al.*, 1999). The localization of PAR-2 proteins in *pod-7(RNAi)* embryos (fig.13) resembles what has been observed in *nmy-2* and *mhc-4* mutants. These data suggest that the lack of POD-7 could affect the polarity establishment at the level of the cytoskeleton reorganization. In agreement with this, similar phenotypes have been detected also in other mutants of the POD class (Rappleye C. A. *et al.*, 2002). In the wild type, during relocation of the polarity markers the movements of the actomyosin cytoskeleton give rise to ruffling of the plasma membrane and formation of a pseudocleavage (fig.1 D). Inhibition of actin filament polymerization with the drug cytochalasin abolishes all the cortical activities (Strome S. and Wood W.B., 1983; Hill D.P. and Strome S., 1988; 1990). Mutants with severe abolishment of ruffling and pseudocleavage always exhibit a defective polarization because they are not able to relocate PAR proteins (Severson A.F. *et al.*, 2002; Severson A.F. & Bowerman B., 2003). In *pod-7(RNAi)* embryos, the ruffles are shallow or completely untraceable; the pseudocleavage is often missing (see fig. 9,10, 12,13). These defects may be caused by a failure in the correct movement of the actin cytoskeleton as suggested by the actin independent localization of PAR2 proteins.

Human cells exposed to osmotic shock decrease the total internal content of F-actin (Hallows H.L. *et al.*, 1996). This phenomenon seems conserved through evolution because also in yeast, in case of osmotic stress, actin filaments and cortical actin patches are disassembled (Chowdhury S. *et al.*, 1992). If this mechanism would also be present in *C. elegans*, it would explain why so many mutants display osmosensitivity coupled to defects in polarity. A defective eggshell generates osmotic sensitivity, that, as result affects the actin concentration and the correct establishment of polarity. In order to verify the effects of osmotic sensitivity on cytoskeleton, it would be interesting to study the concentration of actin and its movements under the conditions of osmotic stress.

Among the mutants that display polarity and osmotic defects, many genes (table n.2) code for components of the meiotic machinery. The reason why subunits of the anaphase promoting complex, if knocked down, fail to extrude the polar bodies, establish polarity as well as regulate osmosensitivity, is not yet understood (Rappleye C. A. *et al.*, 2002). APC<sup>cdc20</sup>

carries out a well characterized role in the mechanism that degrades SECURIN to activate SEPARASE. This protein cleaves COHESINs and eventually resolves the chromosomes in meiosis. Nonetheless, several authors have speculated about the possibility that APC<sup>cdc20</sup> may have additional secondary target(s). In *S. cerevisiae* for instance, SEPARASE cleaves Slk19 (kinetochore and spindle protein) in addition to Scc1 (subunit of the chromosomal cohesin) (Sullivan M. *et al.*, 2001). Both cleavages occur simultaneously, and a common regulative system is likely to exist for the two different proteins (Uhlmann F. *et al.* 1999). No additional substrates that the worm protease might have are identified which makes it difficult to make a hypothesis about their connection to the processes of polarity and osmosis (Rappleye C. A. *et al.*, 2002; Shakes D.C. *et al.*, 2003).

The fact that the proteins that link the degradative pathway to the polarization process are unidentified, leaves open the possibility that, by a change of paradigm, this factor(s) might be required for the activation of the synthesis of the eggshell. Thus the creation of a functional eggshell, would guarantee the correct execution of the polarity establishment. This is an attractive idea because the phenomena of eggshell synthesis and meiosis take place almost simultaneously and it would be efficient for the cell to coordinate both processes through the same regulatory machinery. Therefore, APC<sup>cdc20</sup> might regulate chromosome separation in meiosis and, through a second target, could simultaneously activate the secretion of the eggshell components. If our hypothesis is correct, the POD genes coding for the subunits of the anaphase promoting complex, if mutated, should result in a defective eggshell, too. Unfortunately, the osmotic sensitivity render complicated the EM analysis of these mutants eggs and no data about their eggshell are, at the moment, reported in literature.

The capability to regulate the osmotic balance is an essential feature in the development. Identifying the genes that are responsible for the construction of the eggshell is important for the understanding of how this structure provides a mechanical support, affecting further stages of development. In spite of the fact that it has not been shown whether osmotic sensitivity causes failure in the establishment of polarity, the opposite case can be ruled out. Polarity defects do not provoke osmosensitivity and mutants

with defective polarization do not present any problems in their eggshell. For instance, RNAi against *nmy-2*, *mlc-4* or actin are not osmotic sensitive (Gönczy P. *et al.*, 2000; Guo S. and Kemphues K.J., 1996; Shelton C.A. *et al.*, 1999).

#### **4.4 Mutants with defective eggshell fail to properly extrude the polar body**

Numerous mutants in proteins involved in the construction of the eggshell fail to extrude both polar bodies. The two polar bodies are extruded through different mechanisms: the first polar body is inserted in the eggshell and detached from the embryo whereas the second is located on the cell plasma membrane (fig.11 and 17). Data presented in this study demonstrate that the polar body detached from the embryo is inserted in the vitelline layer. Hoechst dye stains this polar body without permeabilization, thus this polar body is placed external to the impermeable lipid-rich layer. In agreement with this observation is, that this polar body is lost after the procedure that removes the vitelline layer (fig.11). This observation indicates that the first polar body is inserted in the most external (vitelline) layer of the eggshell.

A defective eggshell causes problems in the extrusion of the polar bodies. A possible explanation could be that the eggshell actively participates in pinching off the polar body from the embryonic plasma membrane. The detachment of the polar body takes place through a bilateral action: on one side the cell push the polar body out, but simultaneously, on the other side, the just constructed eggshell must be able to retain it. In this model, eggshells that are not properly constructed have no (or have reduced) capability to retain the polar body. The presence of two polar bodies on the plasma membrane of osmosensitive embryos increase the possibilities that of one of them could be re- inserted into the cell. Additionally, an osmotic sensitive embryo is enlarged and fills up the entire eggshell, requiring a supplementary positive pressure to extrude the polar body. In wild type embryos, the polar body is extruded against the pressure of the medium surrounding the cell, whereas in osmotic sensitive and enlarged *pod-7(RNAi)* embryos, this

pressure is extricated against the solid, although improperly constructed, eggshell (fig. 14, 16).

Moreover, if osmotic shock affects actin concentration, it could also cause problems in the contractile ring that completes the extrusion of the polar bodies.

#### **4.5 A properly constructed eggshell is required for correct development of *C. elegans* embryos**

In spite of the fact that the defects in *pod-7(RNAi)* worms are observed already at one cell stage, these embryos were able to form new blastomeres until the onset of the gastrulation. This observation may suggest that a defective eggshell does not severely affect the development of the worms. Nonetheless, if a wild type eggshell is removed after the two cell stages and the embryo is placed in an isotonic medium, the worm is still able to divide and develop until gastrulation, but the phenotypes observed are absolutely aberrant (Schierenberg E. and Junkersdorf B., 1992).

Because the eggshell allows the carrying out of fundamental processes for the development of the worms, POD-7 (and EMB-8) might be excellent targets for designing drugs with nematocide action. These drugs are important, for instance, against the numerous parasite nematodes that affect humans. Indeed, EM data have showed that the three layers of the eggshell are conserved among the different species (Burgwyn B. *et al.*, 2003; Foor W.E., 1967; Bird A.F. and Bird J., 1991). This morphological conservation suggests that enzymes that construct the eggshell might be also conserved, thus a drug interfering with *C. elegans* POD-7's metabolic action could be lethal for other nematodes, too.

#### **4.6 POD-7 might be a model system to study the human Bietti crystalline corneoretinal dystrophy**

The lack of information about lipids is in strident contrast with the increasing evidences that associate the metabolic actions of specific lipids to numerous and severe pathologies.

The human homologue of the enzyme POD-7 is CYP4V2. *Cyp4v2* gene has been found mutated in patients affected by a severe disease called Bietti Crystalline corneoretinal Dystrophy (BCD). BCD is an autosomal recessive retinal dystrophy characterized by multiple glistening of intraretinal crystals. The symptoms of BCD include: crystals in the cornea, yellow and shiny deposits on the retina, and progressive atrophy of the retina, choriocapillaries and choroids (the back layers of the eye) associated with atrophy of Retinal Pigment Epithelium (RPE) (Welch R.B., 1977). In spite of the ubiquitous pattern of expression of CYP4V2, people with BCD accumulate crystals in some specific cells (RPE and lymphocytes). The presence of these crystals leads to progressive night blindness and constriction of the visual field. Researchers have been unable to determine exactly what substance gives rise to these crystalline deposits and, at the moment, there is no treatment for BCD. A possible explanation for the formation of crystals in BCD's patients is that, when CYP4V2 is mutated, its unmetabolized substrate accumulates and precipitates forming crystals. The understanding of the chemical nature of the substrate of CYP4V2's might facilitate the finding of a cure, decelerating or preventing the process of crystal formation.

So far two proteins have been connected to BCD: the already described lipid modifier CYP4V2 (Lee J. *et al.*, 2001; Lin J. *et al.*, 2005; Shan M. *et al.*, 2005; Li J. *et al.*, 2004) and an uncharacterized 32 KDa FABP (Fatty Acid Binding Protein) identified in a FA cross-linkage experiment (Lee J *et al.*, 1998).

If CYP4V2 and the FABP give rise to the same pathology in humans, it would be interesting to test if *fabp(RNAi)* can also lead to defects in the eggshell and to the embryonic lethality observed in *pod-7(RNAi)* embryos. FABP proteins are redundant in *C. elegans*, thus the silencing of the FABP proteins (*lbp-2*, *lbp-4*, *lbp-5*, *lbp-7*, *lbp-8*; lbp stands for Lipid Binding Protein) do not phenocopy *pod-7(RNAi)*. However, the involvement of FABP in the process of eggshell formation (in which POD-7 is involved) is reported for *Ascaris suum*. In this nematode, the protein As-p18 has the capability to bind (and transport) fatty acids contributing to the creation of the lipid-rich layer of the eggshell (Mei B. *et al.*, 1997). All the lipid-modifying enzymes described above, involved in metabolism of fatty acids, present defects in the eggshell. This observation



leads to characterize the composition of this class of molecules in the extracts obtained from *emb-8(hc69)* embryos. Moreover, the data obtained will be compared with the analysis of control worms' extracts. Data contained in this thesis suggest that EMB-8 and POD-7 perform their function in the same metabolic pathway. The comparison of the lipid extracted from *emb-8(hc69)* mutants is at the moment in progress and hopefully will provide the definitive answer about the class of molecules that POD-7 (and perhaps also CYP4V2), together with EMB-8, metabolize.

## 5- MATERIAL AND METHODS

### Worm strains

Wild-type N2 Bristol and mutant strains were routinely propagated on NGM-agar plates as described in (Brenner S.,1974). The following strains were used: N2 (wild type), NL4256 *rrf-3(pk1426)*, MJ69 *emb-8(hc69)III*, DR476 *daf-22(m130)II* all the mutants were obtained from the Caenorhabditis Genetics Center. The temperature sensitive *emb-8(hc69)* allele was maintained at 16°C. Bleached embryos were placed at non-permissive conditions (25°C) in order to produce mutant phenotypes. The following GFP transgenic strain JH1380 (GFP::PAR-2), TH25 (GFP::PAR-6) AZ212 (GFP::H2B) have been kindly supplied from Anthony Hyman.

### RNAi interference by feeding

RNAi by feeding was performed essentially as described in (Fraser A.G. *et al.*, 2000). Briefly, portions of the genomic DNA (0,5-1,5 Kbp) were amplified by PCR (Polimerase Chain Reaction) from genomic DNA with oligonucleotides that inserted restriction sites for NotI (see below). The PCR products were inserted into L4440 DoubleT-7script II vector (Timmons L. and Fire A., 1998). A culture of HT115 (DE3) *E. coli* was transformed and grown overnight on a LB plates with 50  $\mu$ g/mL ampicillin (Timmons L. and Fire A., 1998; Timmons L. *et al.*, 2001). (<http://elegans.swmed.edu/perl/CGCStrain.pl?recnum=2667>). A single colony was inoculated into 50 mL bacterial cultures in LB/Amp and let grow the concentration of  $0.37 \pm 0.02$  OD (600 nm) (6-8 hours); eventually resuspended in 5 mL of LB/Amp, 50  $\mu$ l culture were used for seeding on NGM/Amp/ and isopropyl-beta-D-thiogalactopyranoside (IPTG) of a 12-wells plate (Nunc, Roskilde, Denmark). About 30 bleached eggs were placed on them and the arising phenotype were monitored every eight hours. Oligos used for the

amplification of the cloning were the following (in the table are not listed the linker with the NotI sequence restriction site: **CCGCGGCCGC**):

<b>Gene</b>	<b>ORF</b>	<b>Forward</b>	<b>Reverse</b>
<b>PBR</b>	C41G7.3	AAGTTGCTGATTGGTGGGCG	GTTCTTGATTGGGTCATTCTCC
<b>CYP-44A1</b>	ZK177.5	TCTCAACACCTTCTTGCGCG	GCAAACGGATGAACTTCTTTTCG
<b>Adrenodoxin</b>	Y73F8A.27	CACAATGTCCTCCGTGCTCAG	GGAAAAAATACAACCCGAATCTCG
<b>A Reductase</b>	Y62E10A.6	CCCCTCTCCCCCTCATTTTATG	TTGACATTTTTTCGGCGGC
<b>CYP-31A2</b>	H02i12.8	TTTCCAGCACCTCAATCAGCC	AATGCGAGACGACCTTCCATC

Additional bacterial clones (*emb-8* -K10D2.6, *pod-2* - W09B6.1 , FAS - K10D2.6) were purchased from MRC Geneservice.

### **Live Imaging**

Dissected embryos were mounted for filming on agarose pad. Widefield microscopy on a motorized microscope (Axioplan II; ZEISS) was used to film JH1380, TH25 and Z212 strains. Image processing was done with Meta View Software (Universal Imaging Corporation).

### **Dyes staining for embryos**

Live analysis was performed by dissecting embryos in M9 buffer and Hoechst 33342 10 ng/ $\mu$ l (Molecular Probes, Eugene, Oregon, United States) and FM 4-64 32  $\mu$ M (Molecular Probes, Eugene, Oregon, United States). Embryos were washed briefly with M9 medium, and mounted on agarose pads.

Chitinase treatment was performed as in and after the treatment embryos were placed in minimal EGM to avoid osmotic shock (Edgar L.G., 1995).

### **RNAi interference by microinjection**

Primers to amplify CYP31A2 from the L4440 DoubleT-7script II vector were the same annotated above, with the addition of the sequence for the T7

and T3 RNA polymerases (respectively TAATACGACTCACTATAGG and AATTAACCCTCACTAAAGG). PCR products were purified by using a PCR clean up kit (Quiagen), eluted with 40  $\mu$ l water and used as templates for 25  $\mu$ l T3 and T7 *in vitro* transcription reactions (Ambion). After incubation the transcription reaction for 3-5 hours at 37° C, DNase (1.3  $\mu$ l/reaction) was added and incubated for 15 minutes at 37° C. The product of the reactions were cleaned using an RNeasy kit (Quiagen), and the RNA was eluted in a final volume of 60  $\mu$ l. T3 and T7 reactions were pooled and mixed with 3X injection buffer (60mM KPO<sub>4</sub> pH 7.5, 9 mM K-citrate pH 7.5 and 6% PEG 6000). Annealing was performed by incubating the reaction at 94° C for 10 min, followed by an incubation of 60 min at 37° C. Samples of the T3 and T7 transcription reactions and a sample of the annealed dsRNA was loaded on an agarose gel. Annealed dsRNA exhibited a band shift compared to single stranded RNAs. DsRNA was aliquoted, snap frozen in liquid nitrogen and stored at -80° C.

The dsRNA was injected into L4 hermaphrodites or young adults, the dissected embryos were observed at the microscope after 24 hours.

### **Electron microscopy**

Whole worms were cryoimmobilized using an EMPACT2+RTS (Leica Microsystems) high pressure freezer (Manninen A. *et al.*, 2005). Samples were freeze substituted at -90°C for 2 d in acetone containing 1% osmium tetroxide and 0.1% uranyl acetate (Müller-Reichert T. *et al.*, 2003). The temperature was raised progressively to room temperature over 22 h in an automatic freeze substitution machine (Leica Microsystems). Samples were embedded in epon/araldite and thin sections (70 nm) were cut using a Leica Ultracut UCT microtome. Sections were collected on Formvar-coated copper grids, poststained with 2% uranyl acetate in 70% methanol followed by aqueous lead citrate and viewed in a TECNAI 12 (FEI) transmission electron microscope operated at 100 kV.

### **TLC with of lipid extracted from *C. elegans***

To investigate metabolism in *C. elegans*, 10-cm NGM agar plates were prepared. A quantity of 300  $\mu$ l of bacterial suspension mixed with 5  $\mu$  Ci of [3H]-cholesterol (Amersham Biosciences Europe, Freiburg, Germany) or [14C]Acetic acid (Amersham Biosciences Europe, Freiburg, Germany). Worms were harvested from the plates with M9 medium and subjected to three cycles of freezing-thawing. The lipids were extracted by the Bligh and Dyer method (Bligh E.G. and Dyer W.J., 1957). Extracts were analyzed by TLC performed on glass-backed plates of silica gel 60 (Merk, Darmstadt, Germany). Solvents used for the separation of cholesterol metabolites were chloroform-methanol (24:1). After chromatography, plates were sprayed with a scintillator (Lumasafe, Lumac LSC B.V., Groningen, The Netherlands) and exposed to a film (Hyperfilm MP, Amersham Biosciences Europe, Freiburg, Germany).

### **Preparation and fractionation of a lipidic extract**

L1 population of worms *daf-22(m130)II* and *emb-8(hc69)* were synchronized on plates without food and then moved to ten 14.5 cm Ø plates with bacteria. After three days, gravid worms were collected by rinsing with ice-cold water, transferred into 50-ml Falcon tubes and centrifuged (2 minutes 1000 rpm). After bleaching procedure, the collected embryos were subjected to three cycles of freezing-thawing. The eggs suspension were transferred into a glass bottle with 19 volumes of methanol containing 10  $\mu$ g/ml of antioxidant BHT. The extraction was performed overnight at room temperature under continuous agitation. Extracts were separated from worm remnants by filtration through a Whatman GF/A glass filter. Methanol extracts were combined and extracted two times with one volume of hexane. The obtained hexane extract was washed twice with a methanol-water mixture (9:1), dried under N<sub>2</sub> flow, and dissolved in 10 ml of hexane.

## Bioassay

Testing of the biological activity of lipidic fractions was performed in 12-well cell culture plates (Nunc, Roskilde, Denmark). Each well contained 1 ml of NGM agar mixed with 0.1% tergitol. A quantity of 20  $\mu$ l of hexane or methanol fractions were added per well and dried in the laminar flow cabinet. Because methanol seemed to have an intrinsic toxicity, we dried these extracts in speed *vacuum*, resuspended in beta-isopropanol and then added to the bioassay. Before seeding worms, 50  $\mu$ l of bacteria (prepared as described above) were added to plates and left overnight at room temperature.

Around 30 eggs/well were grown one generation on the bioassay and after 100 hours seven gravid adults were placed on a new plate. These worms laid eggs for about two hours and they were eventually removed. In accordance with the definition of embryonic lethality, after 24 hours worms and eggs were quantified and the activity of fractions was represented as the percentage of hatched L1 vs. total worms in the plate (egg+L1). Duplicates of each fraction per experiment were analyzed.

## 6- ABBREVIATIONS

APC	Anaphase Promoting Complex
BCD	Bietti Crystalline corneoretinal Dystrophy
CGC	Caenorhabditis Genetics Center
CHS	CHitins Synthase
CSG	Chromosome SegreGation
CYK	CYtoKinesis defect
CYP	CYtochrome P450
DAF	DAuer Formation
DGLA	Dihommogamma-linolenic acid
DIC	Differential Interference Contrast
dsRNA	Double Stranded RNA
EGM	Embryonic Growth Medium
EM	Electron Microscopy
EMB	EMByonically lethal
FABP	Fatty Acid Binding Protein
FA	Fatti acids
HPLC	High Performance Liquid Cromatography
HSP	Heat Shock Protein
IPTG	Isopropyl-Beta-D-ThioGalactopyranoside
L1	Larval stage 1
L4	Larval stage 4
LBP	Lipid Binding Protein
NCPR	NADPH Cytochrome P450 Reductase
NGM	Nematode Growth Media
NI	Not Induced
NMY	Non-muscle MYosin
ORF	Open Reading Frame
PAR	abnormal PARTitioning of cytoplasm

PB	Polar Body
PCR	Polimerase Chain Reaction
POD	Polarity and Osmotic sensitivity Defect
PUFA	PolyUnsaturated Fatty Acid
RNAi	RNA interference
RPE	Retinal Pigment Epithelium
TLC	Thin Layer Chromatography
TS	Thermo Sensitive



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## **9- DECLARATION**

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

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